Microalgae for the biochemical conversion of CO2 and production of biodiesel

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Microalgae for the biochemical conversion of CO_2 and production of biodiesel

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ABSTRACT

As the global population rises to an estimated 9.4bn by 2050, the pressure for food, fuel and freshwater will continue to increase. Current renewable energy technologies are not widely applicable to the transport sector, which requires energy dense liquid fuels that drop into our existing infrastructure.

Algal biofuels promise significantly higher yields than plants, without the displacement of valuable agricultural resources and have the potential to meet the global demand for transport fuel. Fossil fuel energy is largely 'a legacy of algal photosynthesis', with algae accounting for ~50% of global CO_2 fixation today. In addition, these curious organisms show remarkable diversity in form, behaviour and composition. Recently there has been a global resurgence of interest in microalgae as a resource of biomass and novel products.

With the present level of technology, knowledge and experience in commercial scale aquaculture, the capital cost and energy investment for algal biomass production is high. Culturing, harvesting and disrupting microalgal cells account for the largest energy inputs with more positive energy balances requiring low energy designs for culture, dewatering and extraction, efficient water and nutrient recycling with minimal waste.

Little is known about the variable cell wall of microalgae, which presents a formidable barrier to the extraction of microalgal products. Staining, transmission electron microscopy (TEM) and enzymatic digestion were all utilised in an attempt to visualise, digest and characterise the cell wall of stock strains of *Chlorella* spp. and *Pseudochoricystis ellipsoidea*. The presence of algaenan, a highly resistant biopolymer, rendered staining and enzymatic digestion techniques ineffective. TEM revealed that algaenan is present in the outer walls of microalgae in a variety of conformations which appeared to impart strength to cells. A preliminary investigation utilising *Fusarium oxysporum* f.sp. *elaeidis* as a novel source of enzymes for the digestion of algaenan has also been described.

Methods were developed for the mutagenesis of *Chlorella emersonii* and *P. ellipsoidea* using EMS and UV with the intent of generating cell-wall mutants. Although no viable cell wall mutants were produced, a viable pale mutant of *C. emersonii* was recovered

from UV mutagenesis. Growth rates of the pale mutant were significantly slower than the wild type, yet FAME profile was largely unaffected. Fluorescence activated cell sorting (FACS) was also investigated as a means for the rapid screening of mutagenized cells for cell wall mutants.

In an attempt to reduce cooling costs of closed-culture systems, temperature tolerant species of microalgae were sought by bioprospecting the thermal waters of the Roman Baths. Numerous methods for isolation and purification of microalgae from the Baths were employed, ultimately yielding seven diverse isolates including cyanobacterial, eukaryotic, filamentous and single celled species. Despite some species possessing an increased tolerance to higher temperatures, none showed marked temperature tolerance coupled with high productivity. Further improvements to the culture conditions may have improved the productivity at higher temperatures. All seven isolates were deposited to the Culture Collection of Algae and Protozoa (CCAP).

A variety of extraction methods including soxhlet, beadbeating, sonication and microwaving was investigated for efficacy of extracting fatty acid methyl esters (FAMEs) from *C. emersonii*. Beadbeating proved most effective in the extraction of FAMEs from *C. emersonii*. Microwaving showed potential as a rapid method of extraction yet was coupled with degradation of FAMEs, requiring further method development to resolve this issue. Method development has been a significant component of the work described in this thesis.

ABBREVIATIONS & ACRONYMS

BBM	Bolds basal medium
BG-11	blue-green 11 medium
BODIPY	4, 4-difluoro-3a, 4a-diaza-s-indacene
bp	base pair
CCAP	Culture Collection of Algae and Protozoa (Oban, Scotland)
DAF	dissolved air floatation
DAPI	4', 6-diamidino-2-phenylindole
DM	diatom medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECF	electro coagulation-flocculation
EMS	ethyl methanesulphonate
FACS	fluorescence activated cell sorter
FAME	fatty acid methyl ester
FT-IR	Fourier transform infrared
GB	Great Bath
GC-MS	gas chromatography mass spectrometry
GHGs	greenhouse gasses
GM	genetic modification / genetically modified
HMDS	hexamethyldisilazane
KB	Kings Bath
OD	optical density
PCR	polymerase chain reaction
PHA	polydroxyalkanoate
PM	pale mutant
PML	Plymouth Marine Laboratories
RNA	ribonucleic acid
S.D.	standard deviation from the mean
SEM	scanning electron microscopy
TAG	triacylglycerol
TEM	transmission electron microscopy
TFA	trifluoroaceteic acid
TLS	trilaminar structure
UV	ultra violet
wt%	weight percent (dry w/w)
w/v	weight/volume (g/l)
w/w	weight/weight (g/g)
v/v	volume/volume (ml/ml)

Ce	*Chlorella emersonii*
Cs	*Coelastrella saipanensis*
Ct	*Choococcidiopsis thermalis*
Cv	*Chlorella vulgaris*
H sp.	*Hantzschia* sp.
K sp.	*Klebsormidium* sp.
Mc	*Microcoleus chthonoplastes*
Ml	*Mastigocladus laminosus*
Os	*Oscillatoria sancta*
Pe ‘obi’	*Pseudochoricystis ellipsoidea strain* ‘obi’
Pe ‘ni’	*Pseudochoricystis ellipsoidea strain* ‘ni’
Sv	*Scenedesmus vacuolatus*

CONTENTS

1. INTRODUCTION

1.1 The fuel crisis

1.1.1 Climate change and energy security

Energy use today is linked with economic prosperity, development and quality of life, with over 13TW of global energy derived from fossil fuels (Parmar *et al.*, 2011). Climate change, fossil fuel depletion and reducing our dependence on foreign oil (Walker, 2009) is driving the need for a change in the way we generate and use energy (McKendry, 2002). In 2008, fossil fuels accounted for 88% of global energy consumption. Greenhouse gasses (GHGs) in the environment have already surpassed dangerously high levels (>450ppm CO_2) and are still rising due to global use of fossil fuels for transport and power generation (Walker, 2009). EU 2020 GHG emissions targets have been set at a 20% reduction, with a 20% increase in energy from renewables. Perhaps most significantly, 10% of vehicle fuels are to be renewable (Pulz *et al.*, 2009).

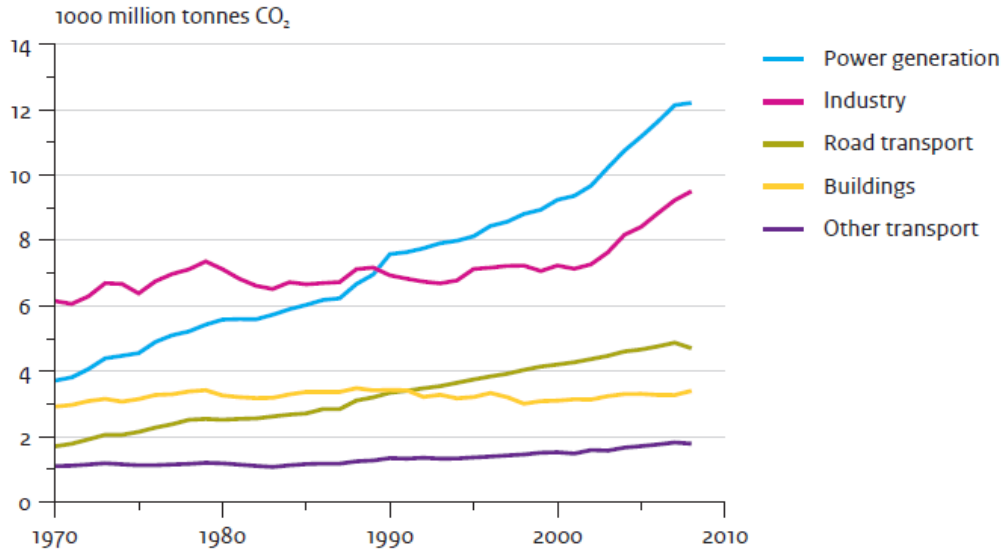


Figure 1.1: Global CO_2 emissions by sector produced from fossil fuel consumption.
Adapted from Olivier *et al.* (2011).

Despite this, almost all new sustainable technologies focus on electricity generation (nuclear, photovoltaic, wind, geothermal, wave, hydroelectric) and do not easily lend themselves to the transport sector (Parmar *et al.*, 2011), the largest consumer sector for fossil fuels (Singh *et al.*, 2011 and Shirvani *et al.*, 2011), requiring energy dense liquid fuels (Figure 1.1). Biofuel is a solution as it could be rapidly implemented into our existing infrastructure.

1.1.2 Traditional biofuels

The energy released from biofuels is derived from biomass. Biomass is a term for all organic matter derived from 'fresh' biological material, either directly or indirectly synthesised from CO_2, water and sunlight via photosynthesis (Figure 1.2). Theoretically biofuels should provide a closed carbon cycle, whereby energy from chemical bonds is released and water and CO_2 are evolved. In 2002 biofuels supplied 10-14% of World's energy supply (McKendry, 2002) and production and consumption is on the increase. The use of bioethanol in the US and Brazil has more than doubled during 2005-2010 from 31.3 to 85.6 billion litres. Biodiesel is currently being produced in smaller quantities but also on the rise from 3.9-18.1 billion litres 2005-2010 (Carriquiry *et al*., 2011).

$$nCO_2 + nH_2O + h\nu \longrightarrow (CH_2O)n + nO_2$$

Figure 1.2: The chemical reaction for the fixation of CO_2 and generation of O_2 via photosynthesis. Barsanti and Gualtieri (2006).

Ideal energy crops have low nutrient requirements, have a high energy yield, of a composition with few contaminants (affecting processing and the final product), yet are low cost and low energy to produce and process (McKendry, 2002). Interesting properties of biomass which affect suitability of a crop for biofuel are; moisture content, calorific value, proportion of fixed carbon, ash residue content and alkali metal content (which can damage processing equipment) and cellulose: lignin ratio (McKendry, 2002). In addition, biofuel research must provide environmentally and economically sound production methods utilising any by-products (Parmar *et al.,* 2011).

Traditional biofuels include higher plants such as oil palm, rapeseed, soybean, sugarcane (first generation biofuels) and waste cellulosic biomass and lignocellulosic crops such as Miscanthus (second generation biofuels). Fermentation of sugary carbohydrate-rich, cellulosic material is used in the generation of biomethane and bioethanol. Biodiesel is produced from biological lipids and of particular interest, as it can be directly implemented into our transport infrastructure. Biodiesel comprises fatty acid methyl esters (FAMEs) produced from transesterification of biological oils or triacylglycerols (TAGs) (Figure 1.3) (Saraf and Thomas, 2007) in the presence of alcohol, high pH and heat (Packer 2009).

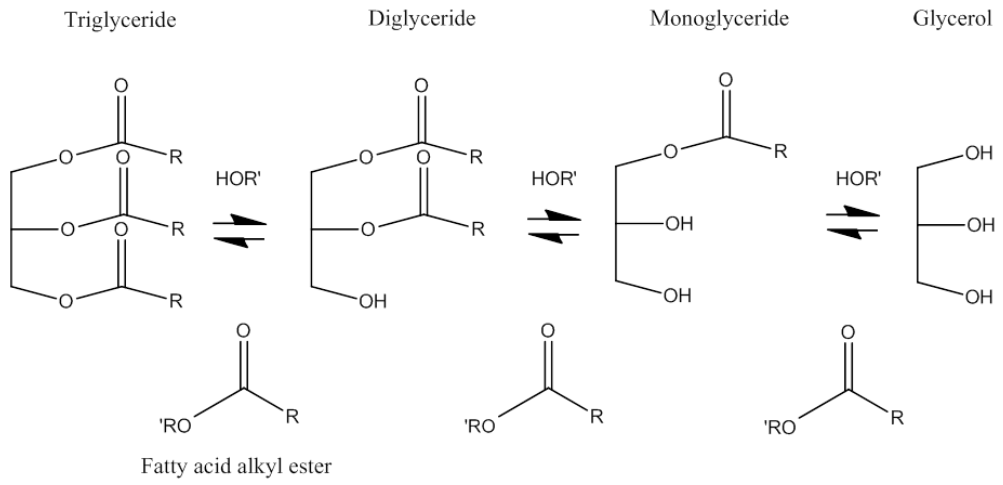


Figure 1.3: Transesterification reaction of TAGs to produce biodiesel (FAMEs) and glycerol.
Adapted from Chuck (2007).

It is not easy to establish the impact of biofuels, hence associated figures are broad and variable in published material. Concerns over traditional biofuel crops (Table 1.1) include high freshwater usage, low yields, poor energy balances and agricultural resource displacement. Localized impacts on climate change due to alteration of land use are usually neglected in life cycle analyses (Reijnders, 2009). Monocultures of lignocellulosic crops tend to sequester less carbon than naturally biodiverse systems and harvest residues (although viewed as waste), are required to replenish the carbon content of soils (Reijnders, 2009).

Table 1.1: Advantages and disadvantages of various biofuel feedstocks.
Carriquiry *et al*. (2011).

Source	Advantages	Disadvantages
Food crops	Established harvesting and processing technologies	Impacts food price by competing for all resources Seasonal harvests
Agricultural residues	Does not impact food prices Avoid changes in GHG emissions due to direct/indirect changes to land use New revenue for farmers	Excess removal will have impact on soil, crop production and environment Needs specially designed harvest equipment
Energy crops	Low water and nutritional input Can grow in poor quality soils Can improve wildlife habitat Reduces soil erosion Improves soil properties Wide geographical distribution Can grow in poor quality soil and dry climate	May compete for agricultural land Seasonal harvests Takes 2-3 yr to reach maximum productivity Consistent high yield unachievable without high input costs Needs development to become optimal Seasonal harvests
Microalgae	High yield potential Can be grown in saline water Can be grown in wastewater Can be harvested all year Does not require arable land	In early stage of development

Advances in technology have brought down the cost of biodiesel (McKendry, 2002), yet agricultural resources for food crops (suitable land, freshwater) are already stretched and according to Walker (2009) 'should under no circumstances be diverted from food toward fuel production'. The initial positivity surrounding biofuels has decreased due to aforementioned disadvantages surrounding resource displacement and their inability to replace fossil fuels. To give an idea of the impracticality of traditional biodiesel crops; in 2008 the UK used 25 billion litres of diesel transport fuel and to supply this as biodiesel from oilseed rape it would require 17 Mha (which represents over 50% of the UK land area) (Scott *et al.,* 2010). In addition, the water footprint for traditional crops is highly unsustainable (Singh *et al*., 2011a) (Table 1.2).

Table 1.2: Land and water requirements for the production of biodiesel, and energy and biofuel yields from various biofuel crops.
Adapted from Singh *et al.* (2011a).

	Water footprint ($m^3 GJ^{-1}$)	**Land use (m^2 GJ^{-1})**	**Energy (GJ ha^{-1} yr^{-1})**	**Biofuel yield (l ha^{-1} yr^{-1})**
Bioethanol				
Cassava	148	79	126	6,000
Wheat	93	305	33	1,560
Rice	85	212	47	2,250
Corn	50	133	75	3,571
Potatoes	105	114	88	4,167
Sugar cane	50	81	124	5,882
Sorghum	180	386	26	1,235
Soybean	383	386	26	1,235
Biodiesel				
Soybean	383	689	15	446
Jatropha	396	162	62	1,896
Rapeseed	383	258	39	1,190
Cotton	135	945	11	325
Sunflower	61	323	31	951
Oil Palm	75	52	192	5,906
Coconut	49	128	78	2,399
Microalgae	<379	2 - 13	793 - 4,457	24,355 - 136,886

These factors have diverged research interests into fuels which are 'non-food', less land and water intensive, which are either integrated into other systems or generate co-products (Carriquiry *et al*., 2011). The relatively recent resurgence of interest in the study of microalgae is arguably largely due to the potential benefits as a biofuel source. Potentially microalgae have all the benefits of plants, coupled with high productivities associated with large scale microbial production. Algae are not only capable of accumulating significantly more lipid than traditional crops (20-80wt%), but also have high biomass yields from non-freshwater systems, do not interfere with food security, do not require arable land, are capable

of utilizing flue gas as a CO_2 source, and could in theory be harvested daily (i.e. non seasonal). Yield of oil from algae is estimated at 20,000-80,000 litres per acre, approximately 7-31 times greater than the most productive oil crop oil palm (Demirbas, 2011a). In addition microalgae boast 'cleaner burning' biodiesel (Mutanda *et al.*, 2011).

1.1.3 Algal biofuels

Between 1978 and 1996 the US Department of Energy invested $25M into the 'Aquatic Species Program' (Ratha and Prasanna, 2012). Comprehensive research was carried out on a variety of species of microalgae, including strain isolation, characterization, biochemistry, engineering and process development. The program was discontinued in 1996 due to reduced federal budgets and lowered petroleum costs. Japan also committed a budget of $117m during this time into researching microalgal utilization of CO_2 (Ratha and Prasanna, 2012). Interest has only recently resurged due to peak oil and limited resources, which is reflected in the infancy of algal research and culture technologies (Pienkos and Darzins, 2009).

Single celled microalgae boast fast growth rates (3.5h doublings are not uncommon) and high oil contents of up to 80wt% (Table 1.3) (Pulz and Gross, 2004). Depending on the species and growth conditions a variety of lipids can be produced including hydrocarbons and triacylglycerols (TAGs) suitable for biodiesel production (Singh *et al*., 2011). Suitable species for biodiesel production must be capable of fast growth rates *coupled with* high lipid content for maximum productivity (i.e. *Botryococcus braunii* has a very high lipid content but very slow growth rates and as such is unsuitable). It is worth noting however, published values for lipid yields may not account for the quantities of lipids suitable for fuel. For example, chlorophyll is unsuitable for biodiesel production, yet often constitutes a significant proportion of extracted lipid from microalgae.

Table 1.3: Oil content present in various species of microalgae.
Adapted from Singh *et al.* (2011), unless otherwise indicated.

Species	Lipid content (wt% (w/w))
*Anabaena cylindrica***	4-7
Botryococcus braunii	25-80
Chlamydomonas reinhardtii	21
*Chlorella emersonii****	25-63
Chlorella protothecoides	14-58
Chlorella vulgaris	14-22
Dunaliella salina	6-25
*Dunaliella tertiolecta ****	16-71
Hantzschia sp.*****	66
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.*	45-50
*Phaeodactylum tricornutum****	18-57
Scenedesmus dimorphus	16-40
*Scenedesmus obliquus****	11-55
*Spirulina platensis****	4-16
Synechococcus sp.**	11

*Demirbas (2011), **Demirbas and Demirbas (2011), ***Varfolomeev and Wasserman (2011) ****Ratha and Prasanna (2012).

Biodiesel is generally heterogeneous, containing chain lengths between C_{16}-C_{24} with varying degrees of unsaturation. The FAME profile is also dependent on the source of the lipids and the conditions under which algae were cultivated (Saraf and Thomas, 2007). Thus biodiesel tends to have variable fuel properties that can substantially affect engine performance (Fortman, *et al.*, 2008). Biodiesel contains a relatively high oxygen content by weight which results in more complete combustion than mineral diesel, resulting in lower CO, particulate matter and hydrocarbon emissions (Song *et al.*, 2008) (Table 1.4).

Table 1.4: Physical and chemical properties of typical fossil diesel and biodiesel in the US in 2006. Adapted from Song *et al.* (2008) and Day *et al.* (2012).

Fuel Property	ASTM, EU standards	Diesel	Biodiesel	Microalgal diesel *
Kinematic Viscosity (40^{o}C)	3.5-5.0	1.3-4.1	4.0-6.0	5.2*
Specific Gravity (kg/l,60^{o}C)	0.86-0.90	0.85	0.88	0.86*
Density (lb/gal, 15^{o}C)		7.079	7.328	
Carbon (wt%)		87	77	
Hydrogen (wt%)		13	12	
Oxygen (wt%)		0	11	
Sulphur (wt%)		0.05	0.00-0.0015	
Boiling point (^{o}C)		180-340	315-350	
Flash Point (^{o}C)	min. 100	60-80	100-170	115*
Cetane Number		40-55	48-65	

ASTM = American society for testing and materials* (Varfolomeev and Wasserman, 2011). EU = European standard (Day *et al.*, 2012).

Microalgae typically accumulate a wide range of TAGs with varying chain lengths, with the bulk (>70%) consisting mainly of FAMEs C_{16}-C_{18} (often $C_{16:1}$ and $C_{18:1}$) (Varfolomeev and Wasserman, 2011), yet are capable of producing a much wider range of FAMEs than any biodiesel crop (Williams and Laurens, 2010). Microalgal oils differ from typical plant oils mainly in having a higher degree of polyunsaturation. This makes it susceptible to oxidation in storage and must be modified to meet international standards prior to use (Pienkos and Darzins, 2009). When subjected to engine tests algal oils typically came close to conventional diesel, with less sulphur (Demirbas and Demirbas, 2011) and better engine performance (Haik *et al.,* 2011).

The FAME profile and content of microalgae can be greatly affected by varying the culture conditions (further discussed in Chapter 1.2.3). Green algae (Chlorophyta) are the most widely studied oil rich group of algae and currently research is being conducted on manipulating metabolic pathways, to produce algae that synthesise 'designer fuels' (Singh *et al*., 2011). However, the tools for the genetic modification and study of algal strains is still under development (Chapter 4.1).

Algae usually accumulate large quantities of storage lipids or non-membrane lipids, when under stress, with doublings in lipid content not uncommon (Illman *et al.,* 2000). Imbalance in C:N ratio inside cells of microalgae affects carbon allocation in cells (Palmucci *et al.,* 2011), diverting carbon from protein and carbohydrate synthesis into oil production (Li *et al.,* 2010), by siphoning off the large NADPH and ATP reserves which would otherwise be used for growth (Thompson, 1996). This is referred to as 'nitrogen starvation'. In some species of green algae (e.g. *Chlamydomonas* sp.), starvation causes formation of male and female gametes (Thompson, 1996). Not all species accumulate lipids under these conditions and not all have the same fatty acid profiles. Algae may also accumulate lipids under other stress conditions such as elevated salinity or high light (Thompson, 1996). Often the lipids are a storage material and used as an energy source when more favourable conditions return. Some species may even use accumulated oils as a means of reducing photosynthesis and absorbing incoming light. Carotenoids can act as 'light shields' which absorb photooxidative radicals.

Algae are capable of producing biofuels other than biodiesel (Figure 1.4). In addition to direct burning of algal biomass, waste biomass can be fermented for the generation of biomethane and bioethanol (Parmar *et al.,* 2011) (Collet *et al.,* 2011) and some species have been shown

to produce hydrogen via activity of a chloroplastic hydrogenase powered by reductive reactions derived from photosynthesis. There are studies currently underway to improve the process so algae produce H_2 under growth conditions (Rühle *et al.*, 2008). A few species have been shown to produce hydrocarbons (similar to kerosene) (Park *et al.*, 2005) and a few cyanobacteria capable of secreting ethanol; *Oscillatoria* sp., *Microcystis* sp., *Cyanothece* sp. and *Spirulina* sp. (Luo *et al.*, 2010). Efficiency may require genetic engineering on multiple parts of the metabolism, which is no easy feat (Bonente *et al.*, 2011), further discussed in Chapter 4.1.

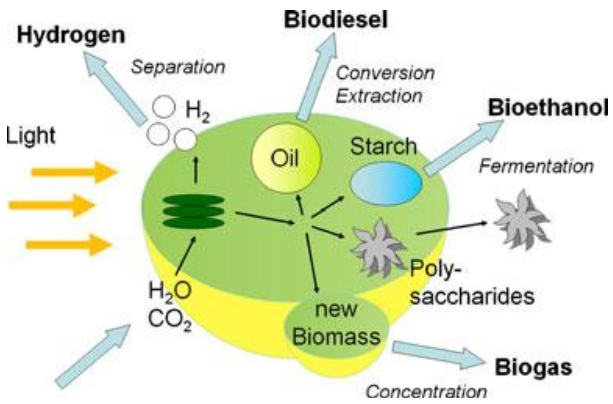


Figure 1.4: Algae offer a variety of biofuels and other valuable products.
In addition to the production of hydrogen, biodiesel, bioethanol and biogas, algal biomass can be burned directly as fuel (Morweiser *et al.*, 2010).

With respect to total land and freshwater use algae perform well, yet currently fare poorer than land crops on energy use and GHG emissions. Cultivation and downstream processing of algae consumes energy in infrastructure and operation (cultivation, harvesting and dewatering, extraction separation, transport, man power and maintenance) (Parmar *et al.*, 2011). The algal energy-footprint is largely due to the power required to run culture systems, CO_2 and nutrient delivery and downstream processing of biomass (discussed in Chapters 6.1 and 7.1) (Singh *et al.*, 2011). However, there is still much to be learned about this diverse group of organisms.

1.2 Algae

1.2.1 The diversity

'Algae' is a term frequently used to refer to a variety of 'aquatic photosynthetic microorganisms' from multicellular macroalgae to unicellular species (microalgae). Unlike plants, algae lack roots, stems, leaves, 'conducting vessels' (xylem/phloem) and complex sex

organs (Croft *et al.,* 2006). The term microalgae is usually a reference to eukaryotic forms, yet is often extended in literature to include cyanobacteria (Mutanda *et al.,* 2011). For simplicity in this thesis, I will be referring to all photosynthetic microscopic organisms as 'microalgae', this will include eukaryotic green algae, diatoms and cyanobacteria. It is however important to remember, that these groups are distinct in their evolutionary history and fundamentally different in their biology.

Microalgae are an incredibly diverse polyphyletic group of organisms with both prokaryotic and eukaryotic representatives (Croft *et al.,* 2006). Between 200,000-800,000 species are believed to exist (Ratha and Prasanna, 2012), yet only a few thousand are kept in collections and a handful cultivated on industrial scale (Parmar *et al.,* 2011). They are all capable of evolving O_2 through the use of photosynthetic pigments and have a range of cell sizes (2-200µm) and feeding strategies (from autotrophic to heterotrophic) (Barsanti and Gualtieri, 2006). Autotrophs simply require light CO_2 and inorganic nutrients for growth, whereas heterotrophs may require an organic carbon source. Previously algae were categorised by their pigmentation, life cycle and cell structure. However many species are highly adaptable in their morphology, which can make this method unreliable (Surek, 2008). As a result, genotypic methods have been employed although these are not yet fully developed (discussed further in Chapter 4.1). Currently algae consist of 11 divisions; 2 prokaryotic groups (Cyanophyta and Glaucocystophyta) and 9 eukaryotic divisions (Rhodophyta, Cryptophyta, Heterokontophyta, Haptophyta, Dinophyta, Apicomplexa, Chlorophyta, Euglenophyta and Chlorarchniophyta) (Croft *et al.,* 2006)) (Figure1.5). The most abundant algae found in environments are the diatoms (Heterokontophyta, (Bacillariophyta)), green algae (Chlorophyta), blue-green (Cyanophyta) and golden algae (Heterokontophyta) (Mutanda *et al.,* 2011).

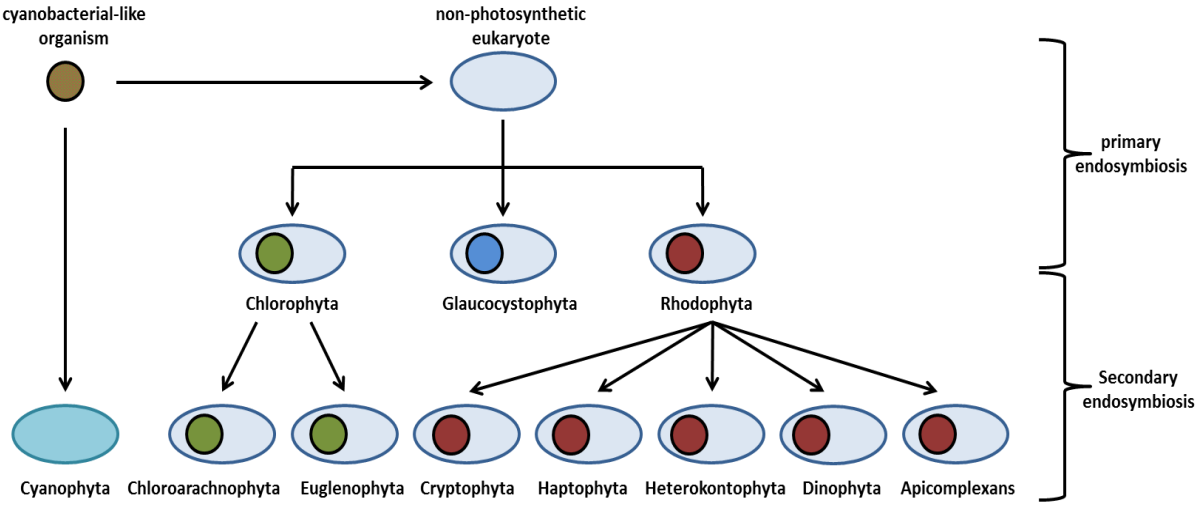


Figure 1.5: The suggested evolutionary history for existent algal groups.
Adapted from Croft *et al.* (2006).

Approximately $2.7x10^9$ years ago photosynthetic bacteria began to synthesise oxygen, and it is from these bacteria that an ancestral cyanobacterium is believed to have evolved (Pulz and Gross, 2004). Cyanobacteria colonise a wide range of ecological habitats, oceans, mountains, hot springs and snow fields (Pulz and Gross, 2004). They consist of (4 orders): Chroococcales (all unicellular species), Oscillatoriales (simple unbranched filaments), Nostocales (Heterocysts), Stigonematales (branched filaments with heterocysts) (Pulz and Gross, 2004). Most cyanobacteria are obligate autotrophs with few growing mixotrophically or heterotrophically and sexual reproduction is absent, as cells replicate by fission (Pulz and Gross, 2004). Those with heterocysts are of particular interest, as these 'specialized' non-photosynthetic cells are capable of fixing N_2, potentially reducing the nitrate requirements (Pulz and Gross, 2004). Cyanobacteria can quickly dominate an environment due to their fast uptake and storage of nutrients (Pulz and Gross, 2004).

The origin of eukaryotic algae can be traced back to ~$1.9x10^9$ years ago which make these algae significantly younger than cyanobacteria. All eukaryotic algae are considered descendants from several endosymbiotic events of a cyanobacteria-like organism into a non-photosynthetic eukaryote (Bozarth *et al.*, 2009). The number of symbioses during algal evolution is heavily debated and is likely to have been several events (Croft *et al.*, 2006), (Figure 1.5).The largest group the Chlorophyceae, is made up of various marine and freshwater forms, unicellular and filamentous with a wide geographical range and fast growth rates (Pulz and Gross, 2004). This group also includes the most commonly studied species

such as *Chlorella* spp., *Chlamydomonas* spp., *Dunaliella* spp. *and Haematococcus* spp. (Pulz and Gross, 2004). Euglenida (commonly found in polluted and waste waters) are flagellated with plant and animal-like characteristics, having arisen from a secondary endosymbiosis between alga and protozoan-like host (Pulz and Gross, 2004). The Rhodophyta have a high phycoerythin content which gives them a red colour, Haptophyta are yellow-green-brown in colour due to xanthophylls and the Heterokonts contain brown algae (Phaeophyta), yellow-green (Xanthophyta), golden algae (Chysophyta) and diatoms (Bacillariophyta), their colours a result of fucoxanthin (Pulz and Gross, 2004). Diatoms are predicted to be the 'largest biomass producers on earth and one of the youngest algal groups, capable of accumulating up to 85wt% in lipids (Bozarth *et al.,* 2009) and due to their high doubling rates, could become a future source of fuel (Pulz and Gross, 2004).

As a result of their complex history, algae are very variable in their biology and physiology (Figure 1.6). Microalgae also play an important ecological role in soil stabilisation and other chemical cycles, their bioactive compounds and extra cellular polymers influencing soil particle adherence, plant growth, water storage and nitrogen fixation (Pulz and Gross, 2004). For example, nitrogen fixation and soil solidification by cyanobacteria (e.g. *Nostoc* sp. or *Anabaena* sp.) is very important in tropical and subtropical agriculture of rice (Grant *et al.,* 1985). It was long believed that algae simply required light, CO_2 and inorganic nutrients in order to grow. However, many species have been found to be growth dependant on vitamin B_{12} (cobalamin), B_7 (biotin) and B_1 (thiamine). Auxotrophy of these vitamins is present in several algal lineages, yet those dependent on external sources often form symbiotic relationships with bacteria capable of synthesising B vitamins (Croft *et al.,* 2006). The majority of algal species are free living, yet a significant number of species are associated with other organisms (e.g. lichens (Sanders *et al.,* 2005), cnidarians, nudibranchs and more recently salamanders (Kerney *et al.,* 2011).

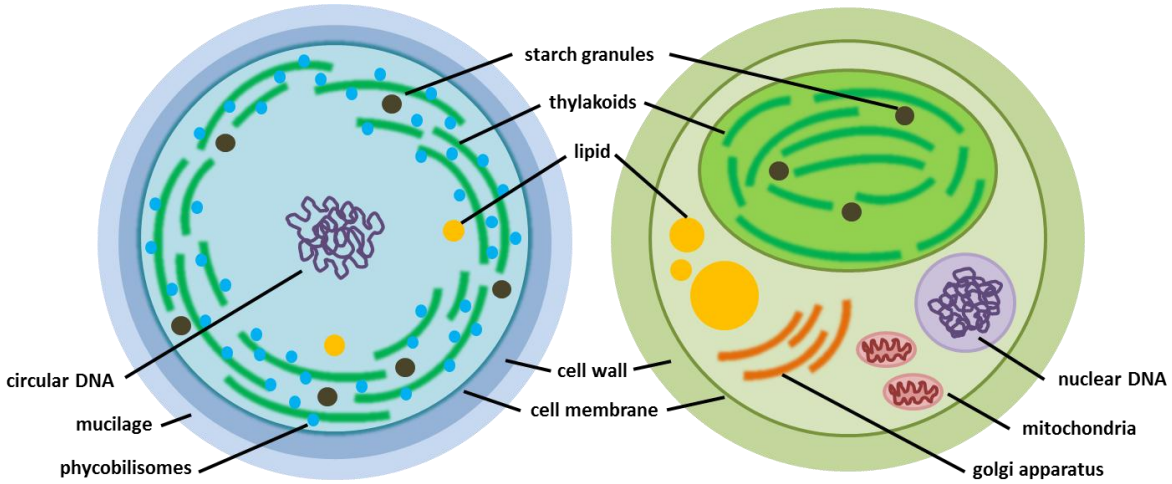


Figure 1.6: Generalised cell structures of a single-celled cyanobacterium (left) and eukaryotic green alga (right).
In the eukaryote DNA and photosynthetic equipment are membrane bound and other organelles are present. Cyanobacteria form a network of thylakoid membranes in the peripheral region of the cell (sometimes referred to as the 'chromatoplast') and contain additional phycobilisomes (light harvesting protein complexes) atop their thylakoid membranes. However in eukaryotic algae thylakoid membranes are interconnected and more stacked, bearing a resemblance to plant cells with small nucleoids of DNA also present in the chloroplast. Cyanobacteria typically accumulate fewer lipids than eukaryotic algae and may possess an additional mucilaginous outer coat. Adapted from Barsanti and Gualtieri (2006).

'Oleaginaceous' is a term used to describe algae capable of accumulating large quantities of oil, >20wt% of their biomass (Karatay and Dönmez, 2011). The most widely studied oleaginaceous microalgal species belong to the Chlorophyta (green algae). This biochemistry of this algal division resembles that of plants, with whom they are believed to share a common ancestor (Thompson, 1996).

1.2.2 Other algal products

Microalgae have the potential to become 'sunlight-driven microbial factories' for many other applications. It is also believed that algal biodiesel may only become economically viable and competitive with the co-production of valuable secondary products (Pulz, 2001). Historically algae have been cultivated as a food source, in particular for the high quality protein content (30-50wt%) (Walker *et al.*, 2005a), containing all 20 amino acids (Singh and Gu, 2010). Today *Chlorella* spp. (2000 t yr^{-1}) and *Spirulina* spp. (3000 t yr^{-1}) dominate the commercial market containing significant quantities of poly-unsaturated fatty acids, omega-3 acids (Pulz and Gross, 2004). Purification of these nutritionally relevant molecules from algal sources could prove more economical than fish liver, without the peculiar taste (Bozarth *et al.*, 2009). Polysaccharides (agar, alginates and carrageenans) are economically important and used diversely in industry for their thickening properties (Pulz and Gross, 2004). Vitamin B_{12}

(cobalamin) is crucial to human health and as plants cannot synthesise this compound, vegetarians and those with autoimmune disorders often have deficiencies (Croft *et al.,* 2005). Many cyanobacteria are capable of its synthesis with *Porphyra yezoensis* (nori) containing as much as liver (Croft *et al.,* 2005), yet there is evidence to suggest that B_{12} in algal food supplements are poorly absorbed by humans (Kumar *et al.,* 2010a).

However, algae derivatives have been shown to positively affect the physiology of animals and are common additives in animal feed and aquaculture (Pulz and Gross, 2004). Such an example is the antioxidant astaxanthin (from *Haematococcus pluvialis*) used in aquaculture for improving the colour of fish (Pulz and Gross, 2004), (Monsant *et al.,* 2001). Pigments such as β-carotene (from *Dunaliella* sp.), phycobiliproteins, phycocyanin and phycoerythin are not only used in food but also cosmetics. Throughout their evolutionary history algae have been exposed to environmental oxidative stresses, and as a result have developed very effective protective and reparative mechanisms, antioxidative complexes and numerous bioactive compounds (neurotoxins, antivirals, immunomodulators and inhibitors and cytostatics), which may have therapeutic effects (anti-cancer, antimicrobial and antiviral activities (Pulz and Gross, 2004)).

Petrochemically derived plastics take decades to degrade in nature, producing toxins and diminishing petrochemical reserves during processing (Suriyamongkol *et al.,* 2007). Polyhydroxyalkanoate biopolymers (PHAs) are being considered as a replacement for conventional plastics. They are completely biodegradable (forming only carbon dioxide and water) making them very attractive for medical applications such as gauzes, osteosynthetic materials and matrices for slow release drugs (Suriyamongkol *et al.,* 2007). They accumulate as a storage granules (0.2-0.5µm in diameter) in the cytoplasm of >300 bacterial species (Spiekermann *et al.,* 1999) and have been found in several cyanobacterial species (e.g. *Synechocystsis* sp. *Spirulina* sp., *Oscillatoria* sp., *Synechococcus* sp.) (Miyake *et al.,* 2000). Similar to the conditions required for lipid accumulation, PHA synthesis can be induced by stressing cells either by nutrient starvation or increasing salinity (Shivastav *et al.,* 2010). Algae are reported to accumulate lower quantities of PHAs as a proportion of dry weight. This may be due to the thick cyanobacterial cell walls preventing efficient extraction of the product or incomplete removal of water during freeze drying (Sudesh *et al.,* 2001). Polyhydroxybutyrate (PHB) is the most common form and its physical properties can be altered drastically by the incorporation of other monomers (such as hydroxyvalerate (PHV))

to form versatile heteropolymers simply by altering the substrate or growth conditions (Sudesh *et al.,* 2001). The cost of processing biomass for PHA production requires it to be a co-product (Suriyamongkol *et al.,* 2007). However PHA could prove problematic as a co-product of microalgal biodiesel, as it could siphon away resources required for lipid production (Suriyamongkol *et al.,* 2007).

Diatoms with their elaborate silica shell are highly variable in their morphology and play a fundamental role in global silicon cycling (Barsanti and Gualtieri, 2006). Presently they are used in applications ranging from filtering liquids, DNA purification and adsorption of metals (Bozarth *et al.,* 2009). However, biomimetics or manipulation of these nano-structures could have a wide range of technological applications in fields such as microelectronics, sensing, drug delivery systems, catalysis and energy storage (capacitors) (Bozarth *et al.,* 2009).

1.2.3 Algal culturing

The first records of macroalgal culture for the production of agar, can be traced back to Japan 1658, yet the first records of microalgal species cultivation of *Chlorella* sp. and *Spirulina* sp. appeared in 1965 (Pulz and Gross, 2004). Algae have basic requirements in comparison to terrestrial crops, yet these vary significantly depending on the species. Algae typically require nutrients and CO_2 at appropriate concentrations, stable temperatures and sufficient light (Barsanti and Gualtieri, 2006). Alteration of these conditions not only influences growth rate but can also affect cell composition and the quantity and quality of products within (Illman *et al.,* 2000). These factors greatly influence the design of a commercial scale plant, something which may require bespoke designs for a given species, application and location (Parker, 2012). For example, *Haematococcus pluvialis* cells are highly shear sensitive (requiring low shear force mixing devices such as an airlift bubble column) and are also light sensitive (with optimal growth occurring at 20μmol photons $m^{-2}s^{-1}$) with high light used for inducing stress and the accumulation of astaxanthin, a valuable antioxidant (Kaewpintong *et al.,* 2007). At present there are two general types of culture system; open ponds and closed photobioreactors (PBRs), each with their own strengths and disadvantages (Table 1.5).

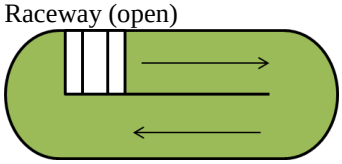
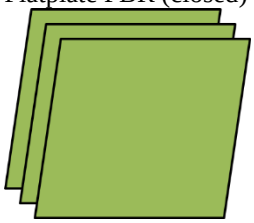
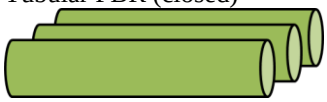
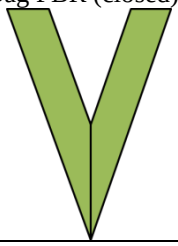
Table 1.5: A comparison of various parameters for open and closed systems.
Adapted from Pulz (2001).

Parameter	Raceway pond (open)	Photobioreactor (closed)
Contamination risk	High	Low
Space needed	High	Low
Water loss	High	Low
CO_2 loss	High	Low
Reproducibility	Low	High
Process control	No	Yes
Standardisation	No	Yes
Species range	Narrow	Broad
Biomass concentration	0.1-0.2g l^{-1} (low)	2.0-8.0g l^{-1} (high)
Harvesting cycles	6-8 weeks	2-4 weeks
Efficiency of treatment	Low efficiency and slow due to low concentration of biomass	High efficiency and quick, small volume flows

Outdoor ponds are significantly less productive than closed PBRs, due to the inability to readily control variables (Pulz, 2001). Extreme conditions (e.g. high salt or pH) are often required to keep out grazers and competing algae and these conditions do not usually infer optimal growth (Reijnders, 2009). Open ponds also appear to have less scope for improvement and innovation than closed photobioreactor systems (Pulz, 2001). Despite this, the largest algal production systems in use today are open ponds, due to their low capital and running costs (Morweiser *et al.*, 2010).

The production of high value compounds is better suited to a closed system, as this allows for reproducible conditions and controllable parameters (Pulz, 2001). In addition, closed systems can achieve high cell densities, 'cleaner' cultures and have reduced water consumption (due to reduced evaporation) (Pulz, 2001) and come in a variety of designs. Abundance of CO_2, light, mixing and temperature control are all important in PBR design. Yet communities in the field are divided on which systems are best for growth, further development and scale up (Norsker *et al.*, 2011). There are three common reactor designs; flat plate, tubular and bag, each with their own advantages and disadvantages (Table 1.6). Scale up is far from straightforward. A company in Wolfsburg Germany took 3yrs to successfully scale up a PBR (to 700m^3) for the commercial production of *Chlorella* sp. (producing 130-150t yr^{-1} of dry biomass per 10,000m^2 of greenhouse) (Pulz, 2001). The time required to 'payback' the carbon and economic capital investment of any PBR is often very significant for any PBR design (Reijnders, 2009).

Table 1.6: A comparison of common algal culture systems.
Adapted from Wijffels and Barbosa (2011), and Singh *et al.* (2011).

Culture system	Light utilisation	Temperature control	Gas transfer	Mixing	Sterlility	Scale up
Raceway (open) 	ok	none	poor	ok	none	difficult
Flatplate PBR (closed) 	excellent	excellent	high	good	easy	difficult
Tubular PBR (closed) 	excellent	excellent	low-high	good	easy	reasonable
Bag PBR (closed) 	ok	good	low-high	poor	easy	difficult

If it were possible to capture 100% of the Earth's incident solar energy, only 0.017% of the Earth's surface would be required to capture the quantity of global energy used today (Scott, 2012). However only a fraction of solar radiation is photosynthetically active and can be utilised by algae (400-700nm). Theoretical photoconversion efficiencies reflect the capacity for an organism to chemically store captured energy. These values have been calculated for algae (9%), which are more simplistic and hence efficient than land plants (4.5%) and make excellent photosynthetic models (Morweiser *et al.*, 2010). Energy losses are primarily due to cell surface reflection, carbohydrate synthesis, respiration and repair (Morweiser *et al.*, 2010).

Light is often the rate limiting factor in most culture systems (Kaewpintong *et al.*, 2007) and is often the primary factor in the design of culture systems potentially influencing the choice of species (Walker, 2009). Photosynthesis comprises of a series of light dependent (light) reactions (not affected by temperature) and a series of light independent (dark) reactions

(affected by temperature) (McKendry, 2002) (Figure 1.7). Light dependant reactions require light to activate photosynthetic pigments which act as a catalyst for the generation of ATP and NADP (Barsanti and Gualtieri, 2006). Light independent reactions (Calvin Benson Bassham (CBB) cycle) then utilise generated ATP and NADPH to fixate carbon (Barsanti and Gualtieri, 2006).

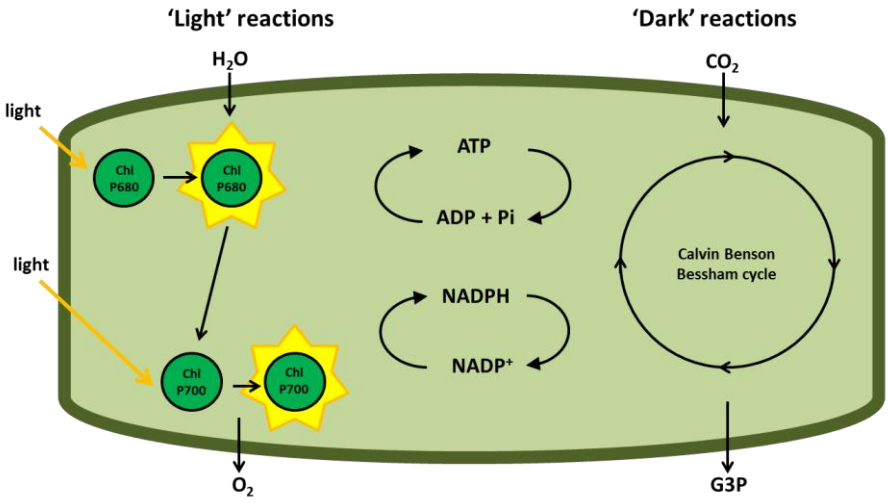


Figure 1.7: Photosynthetic light-dependent and light-independent reactions present in photosynthetic machinery and their involvement in carbon fixation.
Adapted from Melis (2009) and Barsanti and Gualtieri (2006). G3P = glyceraldehyde 3-phosphate.

Microalgae can quickly become light saturated resulting in 'photoinhibition', a well-documented process associated with partial deactivation of the photosynthetic system and often extensive cell damage by the production of free radicals (Gordon and Polle, 2007). The cell then has to expend energy in order to repair the damage (Camachorubio *et al.*, 2002). High frequency alternation of light-dark cycles ('flashing') on millisecond scale can reduce photoinhibition and accelerate growth significantly (Morweiser *et al.*, 2010), (Gordon and Polle, 2007). As cultures become more dense and cells begin to shade each other ('mutual shading'), productivity is slowed (Pulz, 2001). This has prompted the generation of low chlorophyll mutants, which have exhibited superior productivity to wild-type controls (Melis, 2009). Tubular reactors have a circular profile, which has been shown to counterbalance mutual shading (Morweiser *et al.*, 2010). However, changing the orientation of flat panel or tubular PBRs may also have a significant effect on light angle and delivery to a culture (Wijffels and Barbosa, 2011).

Thorough mixing of cultures is required for optimal gas exchange, light delivery and dispersion of nutrients (Wijffels and Barbosa, 2011). Bubble column and airlift designs combine mixing and aeration via delivery of air at the bottom of the culture system (Garcia Camacho *et al.,* 2001). The specific shape and surface area of a reactor greatly affects the energy required for mixing (Wijffels and Barbosa, 2011). For example, the approximate energy input for plate and airlift PBRs is ~50-60 W m^{-2} and yet up to ~2000 W m^{-2} for tubular PBRs (Love, 2011). High gas velocities are linked to high productivity, however can cause significant mechanical stress to the microalgal cell wall (Pulz, 2001). Bubble formation at spargers is often responsible for cell death due to shear stress, affecting productivity (Pulz, 2001). Supplementation of cultures with carboxymethyl cellulose (modifier of interfacial properties) has shown to protect against shear damage (Camacho *et al.,* 2001). Mechanical agitation however, has been shown to be cause more cell damage (Barbosa *et al.,* 2003).

It is estimated that on average 1.65-3.00g of CO_2 is required per gram of algal biomass (Morweiser *et al.,* 2010). Many algae require a CO_2 enriched gas supply for optimal growth rates (Morweiser *et al.,* 2010). However, pH is affected by aqueous CO_2 and must be controlled within a culture, as it may not only directly damage the algae but can considerably affect liquid chemistry and nutrient availability (Kunjapur and Eldridge, 2010). It is also important that generated O_2 is removed in order to push the RuBisCO cycle to 'prepare' CO_2 for the CBB cycle (Figure 1.6) and to avoid the formation of oxygen radicals, detrimental to growth (Barsanti and Gualtieri, 2006). Many algae cannot survive under elevated levels of O_2 for more than 2-3h (Pulz, 2001).

Although algae may have a wide growth range optimal growth temperatures are usually in a narrow range specific to each strain (Kunjapur and Eldridge, 2010). When light or CO_2 is limiting, temperature has little effect on photosynthesis. However under otherwise ambient conditions as temperature increases from 0-50ºC, respiration will rise exponentially (Walker, 2009). Increased temperatures also decrease the solubility of gases in culture media, leading to suboptimal gas exchange (Pulz, 2001). Temperatures can reach as high at 30°C above ambient in a closed PBR without cooling equipment (Morweiser *et al.,* 2010). Seasonal and daily fluctuations ('temperature spikes') can interfere with algal productivity and in highly irradiated regions (e.g. Australia) (Morweiser *et al.,* 2010). Tropical locations may have daily spikes in temperature from 25-45°C, lethal to many microalgae (Huang *et al.,* 2012). As such, cooling of cultures is likely to become a critical parameter of the process (Morweiser *et al.,*

2010). Using evaporation or closed cooling systems removing heat from system increases the energy demand of the process (Morweiser *et al.,* 2010). However, the financial and energetic cost of a temperature controlled system is highly dependent on the PBR design, algae strain and location of the plant (Morweiser *et al.,* 2010).

Growth medium for algae must contain several elements. Phosphorus is essential for synthesis of DNA, RNA, membranes and ATP with starvation being very detrimental to microalgal metabolism (Wang *et al.,* 2008). Absence of sulphur impedes protein synthesis, and inactivates PSII repair system. Mg is needed for nitrogenase activity, Fe is required for ferredoxin, and the formation of NADP (Wang *et al.,* 2008). Trace metals are also essential for photosynthesis (e.g. Mn and Ca are important in H_2O dehydrogenation and O_2 evolution (Wang *et al.*, 2008). Biofilms tend to occur with suboptimal growth conditions, as microalgae 'create' a preferable 'microclimate' (Pulz *et al.,* 2009)

Approximately 45% of the energy that invested into the production of algal oils is in the form of nitrogen fertilisers (Greenwell *et al.,* 2009). With each kg of algal biomass requiring 2kg of CO_2 and 80g of nitrogen, this translates as 8% of the energy of 'fixed' CO_2 is required simply to supply the nitrogen (Greenwell *et al.,* 2009). It should also be noted that at present, nitrogen is worth more than oil ($1.4 kg), with current prices at some $0.4 kg for oil. Nitrogen fixation can be carried out by a number of micro-organisms, including some cyanobacteria (Barsanti and Gualtieri, 2006). However, not many cyanobacteria have been found that accumulate large quantities of oil (Parmar *et al.,* 2011). The provision of nitrogen and phosphorus could be provided by nutrient wastes, the most common of which originate from waste water treatment, anaerobic digester fluids or even mineralized by-products of algae after the oils have been extracted (Greenwell *et al.,* 2009).

Many algae accumulate desired products (i.e. lipids or antioxidants) under stress, which can be induced by altering the culture conditions (Cha *et al.,* 2011). Nitrogen starvation (or change in the C:N ratio) increases the accumulation of storage products in many microalgae (Gardner *et al.,* 2011) as does increasing pH or salinity (Scragg *et al.,* 2002). Higher temperatures and elevated CO_2 levels can increase the saturation of lipids and the remaining sugars can also play a role in determining the types of lipids (Thompson, 1996). Heterotrophic growth of algae has been reported to achieve high cell density on a large scale (Chen and Johns, 1994) and can increase the saturation of storage lipids compared to autotrophic cultures

(Perez-Garcia *et al.,* 2011). Heterotrophic growth of microalgae on crude glycerol (a by-product of biodiesel) has been shown. Glycerol is expensive to purify for industrial use but could become a valuable heterotrophic carbon source (Scott *et al.,* 2010).

Seawater would become an economical way of cultivating algae, as it already contains the majority of nutrients required for microalgal growth (Parmar *et al.,* 2011). Many studies have highlighted the necessity of recycling water or using seawater due to limited freshwater resources (Yang *et al.,* 2011). Seawater however, would require filtering for removal of viruses which may infect an algal culture, reducing its productivity, this would add to capital and running costs (Purton, 2012). Algae can be successfully cultivated using waste water, giving them great scope for integration with waste management, eliminating the need for expensive culture media (Chapter 3.1) (Carriquiry *et al*., 2011). Culturing could also be coupled with scrubbing of CO_2 from industrial flue gas if a source is located nearby (Parmar *et al.,* 2011). The impact of local policies and regulations for water and waste management may greatly affect to development of algal biofuels in some localities (Subhadra, 2011).

There are challenges to modelling of different algal culturing biomass systems due to the biological nature of microalgae (Bernard, 2011). One of the key factors currently limiting the large scale production of microalgae is the often unpredictable behaviour of algae cultures. Despite being unicellular, a population of a single species has complex signalling, which can influence behaviour of other cells. These chemical based communication signals or 'infochemicals' may be harnessed for our benefit in culture control. For example algal grazers can generate short lived aldehydes causing groups of aggregated immotile diatoms to become motile. With the correct trigger 'infochemicals' could be used to initiate lipid accumulation (Cadwell, 2012).

1.2.4 Processing algal biomass

Perhaps the largest challenge regarding process modelling and cost estimation of microalgal products, is the uncertainty regarding downstream processing (Williams and Laurens, 2010). Currently the largest cost in processing algal biomass is 'dewatering' (Wyatt *et al.,* 2012) which can account for 30% of the production cost (Parmar *et al.,* 2011). This is a consequence of the dilute and neutrally buoyant nature of microalgal cultures, which typically reach a cell density of between 10^3-10^8cells ml^{-1} (Pulz, 2001), or 0.1-4wt% (Wyatt *et al.,* 2012). For each kg of raw algal biomass, approximately 1000kg of water must be processed (Parmar *et al.,*

2011). Producing concentrated biomass with current technology requires a large energy input, significantly adding to costs and upsetting the energy balance (Uduman *et al.,* 2011).

Currently dewatering uses a few steps to concentrate cultures into a slurry or paste. Compared to other suspended particles, the variable characteristics of algal cells may require different treatments for different applications (Uduman *et al.,* 2010). Characteristics of algae which impact treatment are: morphology, motility, surface charge, cell density, and extracellular organic matter (EOM) composition and concentration (Henderson *et al.,* 2008). Major techniques such as centrifugation, flocculation, filtration, screening, gravity sedimentation, flotation and electrophoresis, may differ in suitability for a particular species or application (Table 1.7).

Table 1.7: A comparison of water removal and energy use for various microalgal dewatering techniques. Modified from Uduman *et al* (2010).

Method	**Max. water removal (conc. factor)**	**Energy usage (kWh m^{-3})**	**Reliability**	**Limitations**
Flocculation	200-800	Low (varies)	High	Expensive flocculants, may need purification
Centrifugation	120	Very High (8.00)	High	High energy input
Gravity sedimentation	16	Low (0.10)	Low	Process is slow
Filtration	15-60	Low (0.40)	Medium	Filters need replacing
Pressure filtration	50-245	Low (0.88)	High	Filters need replacing
Tangential flow filtration	5-40	High (2.06)	Med-High	High energy, filters need replacing
Flocculation-floatation (DAF*)	n/a	High (10-20)	Med-High	Electrodes need replacing
Electrocoagulation	n/a	Medium-High (0.80-1.5)	High	Electrodes need replacing
Electrofloatation	300-600	Very High (n/a)	High	Electrodes need replacing
Electrolytic flocculation	n/a	Low-Medium (0.33)	High	Electrodes need replacing

*DAF = dissolved air floatation.

Centrifugation whilst efficient (80-90% recovered in 2-5min), is highly energy intensive and therefore not suitable for the production of a biofuel (Uduman *et al.,* 2010). Filtration of larger or filamentous species of algae would be economical, requiring filters with larger pore sizes giving faster flow rates (Uduman *et al.,* 2010). However filtration is often too expensive for small celled species (2-50µm diameter), due to the fine filters and regular backwashing required to prevent blockages (Wyatt *et al.,* 2012). Tangential flow filtration involves recircling of retentate across a membrane, to keep cells in suspension and minimize fouling, useful for shear senstive algae (Uduman *et al.,* 2010).

Flocculation (the aggregation of algal cells) is applicable to many species and large culture volumes (Uduman *et al.,* 2010). The majority of algal cells are 5-50µm diameter with a stable electronegative surface charge between pH 2.5-11.5 (Uduman *et al.,* 2010). Flocculants may be inorganic (ferric sulphate, alum, lime) or polymeric (i.e. Purifloc, Zetag 51, chitosan) and work by charge neutralization and particle 'bridging'. Varying the charge, charge density and length of biopolymers make polymeric flocculants much more versatile. Nature of the flocculant, concentration, algal species and in particular culture pH, can have an effect on efficiency (Uduman *et al.,* 2010). The addition of flocculants has great potential, however depending on the end use of the product may require subsequent separation of the flocculant from harvested biomass (Wyatt *et al.,* 2012), in particular for biodiesel or neutraceuticals whereby toxic or expensive elements may interfere with further downstream processing or final product quality (Uduman *et al.,* 2010). As a result there is great interest in the use of chitosan as a flocculant as it has low toxicity and is easily biodegradable (Cheng *et al.,* 2007).

Autoflocculation of microalgal cultures is highly desirable, and often associated with an increased pH or excreted macromolecules (Park *et al.,* 2011). Microalgae are capable of altering their cell wall composition in response to their environment (Cheng *et al.,* 2007). A high pH can cause autoflocculation in *C. vulgaris*, with the presence of magnesium hydroxide appearing essential for initiation (Vandamme *et al.,* 2012). A novel study by Lee *et al.* (2009) found that under stress, symbiotically cultured microbes produced an extracellular polymeric material which encouraged flocculation of *Pleurochysis carterae.* Another recent study has found that the co-culturing of *Nannochloropsis oceanica* with a bacterium causes efficient flocculation without affecting lipid content of the microalga (Wang *et al.,* 2012). Stress can also induce flocculation in cultures of *Scenedesmus vacuolatus* (data not shown). Algae are capable of regulating their buoyancy according to various stimuli and have been shown to settle differently under light and dark conditions (Harith *et al.,* 2009), such factors could have an effect on floc sedimentation. Alterations to the cell wall of microalgae can greatly affect the efficacy of flocculants and alterations in culture conditions can be employed to improve the efficiency of flocculation (Cheng *et al.,* 2007).

Flotation is a form of physio-chemical separation, whereby small particles <500µm (e.g. algal cells) attach to the surface of microscopic gas bubbles generated in the suspension, (achieved by forcing though small nozzles) which 'float' to the surface of a suspension and can be skimmed off (Uduman *et al.,* 2010). Dissolved air floatation (DAF) is commonly used in

industry and effluent treatment, when used with algae it is often combined with chemical flocculation (Uduman *et al.,* 2010). Electro coagulation-flocculation (ECF) involves the dissolution of reactive anode electrode to form metal cations that neutralise negatively charged microalgal cells (Uduman *et al.,* 2010). The process is strongly improved by high turbulence, high cell densities, decreased pH and choice of anode metal (i.e. aluminium (Al^{3+}) > iron (Fe^{3+})) (Uduman *et al.,* 2010). ECF is best suited to marine species as saltwater lowers power input required (Vandamme *et al.,* 2011). Electrolytic floatation generates bubbles of H_2 at the cathode, creating and carrying microalgal flocs to the surface (Uduman *et al.,* 2010).

Cell size and cell wall structure present a significant energy barrier to product extraction (Razon and Tan, 2011), often requiring mechanical disruption to extract valuable biomolecules within (Parmar *et al.,* 2011). Physical methods for rupturing cells include freeze thawing, grinding, pressing, beadbeating and homogenisation; and chemical methods include solvent extractions, supercritical fluid extraction and direct transesterification (Parmar *et al.,* 2011). All techniques typically require dry biomass to increase efficiency (Dragone *et al.,* 2010), which in the case of microalgal biomass comes with a high energy penalty. One way to reduce the energy input is to use methods suitable for use with wet biomass (Scott *et al.,* 2010), yet osmotic shock is largely inefficient (Parmar *et al.,* 2011) and ultrasound is expensive and energy intensive (Dragone *et al.,* 2010). Enzymatic extractions although attractive are unlikely to ever become industrially viable due to cost, yet can give valuable clues as to the structure and composition of the cell wall (Parmar *et al.,* 2011) (further discussed in Chapter 3). Extraction methods are further discussed in Chapter 7.

1.3 Barriers to 'Oilgae'

1.3.1 The knowledge gap

It is imperative that the energy balance and CO_2 emissions for a biofuel crop must be considered (Shirvani *et al.,* 2011). Algae literature published in the last 3 decades has risen exponentially, with the majority of published data arising out of the USA, China, Germany, UK, and Japan (most published in Bioresource Technology, Journal of Applied Phycology and Biotechnology and Bioengineering) (Konur, 2011), but with research interests worldwide (Chisti and Yan, 2011). Research topics include algal species characterisation, biomass production modelling, design and operation of biomass production systems, assessments of

regional synergistic opportunities, use of domestic wastewater, hydrothermal and catalytic processing of biomass and oil, residual biomass for energy recovery and economic and environmental impacts (LCA, net energy recovery, and production economics) with reviews and analysis of previous publications (Chisti and Yan, 2011). Algae derived biofuels have been argued to produce only as much energy as the fossil fuels require to make them (van Beilen, 2010), (Walker, 2009). However, many studies often do not accurately take into account large scale aquaculture and overestimate the fossil energy input (Chisti, 2008).

There is also significant 'hype' of algal biofuels making claims that often exceed the realistic physical and thermodynamic constraints (Stephens *et al.,* 2010). Many studies and reports make claims as to the low energy balances attributed to parts of their described systems. These are often due to unreasonable extrapolation from data and do not account for many 'hidden' energy costs. For example, a study modelled algal productivity as a function of total annual solar irradiance (Weyer *et al.,* 2010). Unsurprisingly plants closer to 0^{o} were calculated as more productive (approximately twice as productive as 60^{o} latitude) however, the effects of seasonal and daily temperature changes (which greatly affect rates of carbon fixation) were not taken into account (Morweiser *et al.,* 2010). Such hidden costs can considerably add to the energetic, environmental and economic price of biofuel. Common hidden costs include insecticides, herbicides transport of final product and most importantly of all fertilisers (Walker, 2009). In addition dissolved organic matter exuded by microalgae in culture, results in a significant loss of biomass (fixed carbon) (Hulatt and Thomas, 2010).

Industrial algal cultivation is still largely in the R&D phase and assessing costs is challenging as there are many ways to culture and process microalgae which are often species dependant (Carriquiry *et al*., 2011). As such, accurate determination of energy inputs is not straightforward at this stage of research and is still largely subjective. Literature values and methods for TAGs have not been standardized so claims must be viewed with skepticisim (Pienkos and Darzins, 2009). The wide range of values can be attributed to different assumptions and uncertainties in economic and process inputs (Walker, 2009) (Figure 1.8 and 1.9). A 'harmonisation study' was carried out to address this problem, bringing together four partners from research, academia, and industry to estimate algal oil production costs under a common framework (for a variety of plant designs and parameters) and found greatly reduced variability in oil production costs ranging from $10.87-13.32 per gallon (Sun *et al.,* 2011).

Costs however, are still dependant on a number of factors specific to each process design (Parker 2012).

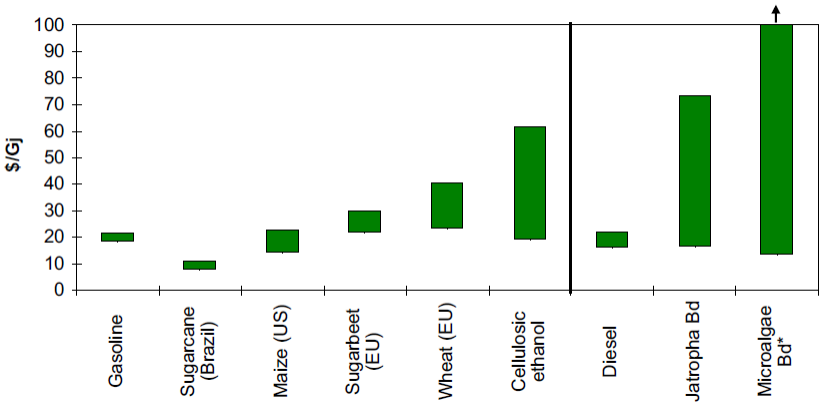


Figure 1.8: Estimated costs of biodiesel from various sources.
From Carriquiry *et al.* (2011). Units in $ per GJ of energy.

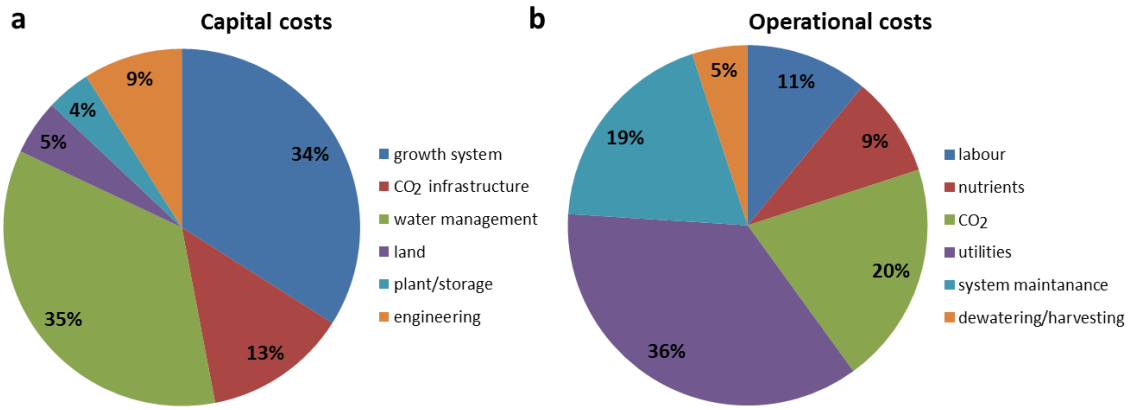


Figure 1.9: Projected costs for a generic 'baseline' microalgal culturing plant, derived using 50 averaged individual parameters from different microalgal culture systems.
(a) capital costs, (b) operational costs. Adapted from Brown (2009).

There appear to be no clear answers to some of the questions posed in this relatively new research field. Conceptually the production of fuel from algae is sound. Research illustrates the need for more in depth and accurate growth models which are species and location specific, in order to produce a comprehensive LCA analysis (Quinn *et al.*, 2011). To make algal biodiesel competitive with fossil diesel it needs to cost no more than $5/gal (Cao, 2009) and the co-production of a more valuable fraction is agreed to be important for commercial success (other algal products are discussed in Chapter 1.2.2). A hybrid biofuel refinery would be ideal to offset energy and economic costs (Singh and Gu, 2010). In the long term GM is

thought to have the biggest impact on fuel from algae (discussed further in Chapter 3.1) alongside advances in dewatering and oil extraction (Chisti and Yan, 2011). It is essential to review all aspects in order to optimize a specific process (Morweiser *et al.*, 2010). As this will cover a range of disciplines, it is vital to encourage collaborations from a variety of sectors including the scientific, legislative and industrial communities (Schlarb-Ridley, 2012).

1.3.2 Creative solutions

Current industrial sized cultivation systems are designed for high value products rather than cheap bulk biomass. Future bioreactors will demand innovative solutions for efficiency in energy and light use, gas transfer and dewatering (Morweiser *et al.*, 2010) (Table 1.8). Research suggests focusing development activity towards technologies which improve algal yield without a cost increase, yet there is great promise in reducing capital costs though novel culturing and harvest technologies (Brown, 2009).

Table 1.8: Summary of proposed solutions to issues surrounding microalgal biodiesel production. Adapted from van Beilen, (2010).

Issue	Potential solutions
Light	Pulsing LEDs, microlensed PBRs
Temperature	IR glass/plastic, use extremophillic species, warmed using cooling water
Nutrient	Wastewater, utilise flue
Dewatering	Filamentous species, self-flocculating/settling species
Oil extraction	GM of excretory pathways
Costs	Co-production of a valuable product, novel PBR designs

Rapid light-dark cycling (>1Hz) can improve photosynthetic efficiency and increase algal productivity (>5-fold compared to continuous illumination) (Love, 2011) by reducing photoinhibition and 'wasted light energy'. This can be achieved with pulsing LEDs which can be further optimized (using light intensity and wavelength) (Gordon and Polle, 2007) or 'microlensing' of the surface of a closed PBR, converting cheap 'continuous' sunlight into a 'stroboscope' (Love, 2011). Both methods have been shown to achieve much higher biomass densities when compared to a normal PBR.

Culture systems that utilise natural sunlight are prone to temporal and spatial patterns of growth due to light or temperature fluctuations (Mutanda *et al.*, 2011). In regions where sunlight is most abundant, increases in temperature can slow productivity (Morweiser *et al.*, 2010). One way to reduce detrimental heating is to block infra-red wavelengths (which constitute 40% of solar radiation), using infra-red reflecting glass and plastic, yet this would

add to capital costs (Stanghellini *et al.,* 2011). In Europe low temperatures provide poor reaction kinetics and slow growth in outdoor culture systems, making them viable for production only between June-October (Sandefur *et al.,* 2011). Warmed cooling water from powerplants could be used to keep cultures warm (Morweiser *et al.,* 2010). Extremophillic algae may be beneficial for industrial applications even if their growth rates are slowed, as contamination risks are lowered if open ponds run hotter (or saltier or at an extreme pH) (Pulz and Gross, 2004). In addition, extremophiles often have unusual metabolites or enzymes which may be of biotechnological interest (Pulz and Gross, 2004). Ecological studies on algal communities may help achieve better yields, as not only are mixed communities more productive (in terms of biomass) but they are also less prone to invasion by airborne microorganisms compared to a monoculture (Smith *et al.,* 2010). Co-culturing may also reduce the nutrient requirements for microalgae. For example, nitrogen biofertilisation using a strain of hyper-ammonium-excreting bacteria (*Azotobacter vinelandii*) has been shown to support the growth of an oil rich green microalga without the need to add nitrates (Ortiz-Marquez *et al.*, 2012).

Savings can be made by coupling algal culturing to other processes. It takes 3t CO_2 to produce 1t of algal biomass (Parker, 2012). Flue gas usually contains CO_2 at 3-15%, with combined cycle gas turbine (CCGT) and coal fired power plants emitting 370t GWh^{-1} and 912-1280t GWh^{-1} of CO_2 respectively (Packer, 2009). The supplementation of CO_2 in microalgal culture increases the rate of growth and biomass accumulation (Packer, 2009, He *et al.*, 2012 and González-López *et al.*, 2012). Warm flue gas may also help raise the temperature of outdoor culture systems. Flue gas however also contains nitroxides, sulphoxides (which can affect pH) and may require filtering prior to use (Packer, 2009). Nutrient removal by algae could be a less expensive and ecologically sound way of scrubbing wastewater (containing nitrates and phosphates), recovering resources, offsetting the cost of fertilizers (Pulz *et al.,* 2009). Phosphorus is vital to any biological growth (synthesis of DNA and ATP) (Wang *et al.*, 2008), yet bioavailable reserves are also being depleted and it is often overlooked as a dwindling resource (Sivakumar *et al.,* 2012). Wastewater treatment is a promising platform for the commercialization of algal growth for biofuels, as domestic wastewater contains nutrients beneficial to microalgal growth (Chistenson and Sims, 2011). Bioremediation of both waste water and flue has yielded some encouraging results. For example, algae cultured in wastewater have shown marked improvements in lipid accumulation when supplemented with CO_2 from flue gas (Devi and Mohan, 2012). However, trace metals can accumulate and

become toxic to algae cultivated using wastewater or flue gas. Yet some studies have shown certain species to have scope for bioremediation of these metals (Bozarth *et al.,* 2009).

Dewatering of dilute microalgal cultures is energy and cost intensive with many established commercial methods being unsuitable for a large facility dealing with enormous effluents (van Beilen, 2010). Filamentous, self-flocculating or settling algal species would be beneficial in greatly reducing costs (van Beilen, 2010). Immobilization techniques already used commercially can often be applied to algae with few modifications. Alginate beads, carrageenan, chitosan and polymers such as polyvinyl foam and polyurethane are suitable materials (de-Bashan and Bashan, 2009). *Scenedesmus dimorphus* has successfully become immobilised onto stainless steel textured metal sheets are textured specifically to allow algal attachment. Sheets are subsequently driven though a conveyor belt to 'mow' the algal lawn, with residual algae serving as an inoculant for subculture (Cao, 2009). Similar promising results have been found using polystyrene as a supporting material (Johnson and Wen, 2010).

Novel, low cost PBR designs can have a large effect on the overall energy demand of an algal culturing system. Many unusual designs are already being used on small commercial scales. For example, the V-shaped bag reactor (Novagreen), flat panel airlift reactor (Subitech Germany) and various designs utilising permeable membranes (Morweiser *et al.,* 2010), (Kumar *et al.,* 2010). One notable design is the Offshore Membrane Enclosure for Growing Algae (OMEGA), which eliminates the use of freshwater and fertilizers by delivering wastewater and flue CO_2 via a permeable membrane system. Additionally the OMEGA system utilises sunlight and is floated offshore, the sea not only controlling the temperature but also mixing the cultures by wave energy (Figure 1.10). Membrane systems do require measures to control the level of fouling and will require replacing periodically (Pulz *et al.,* 2009).

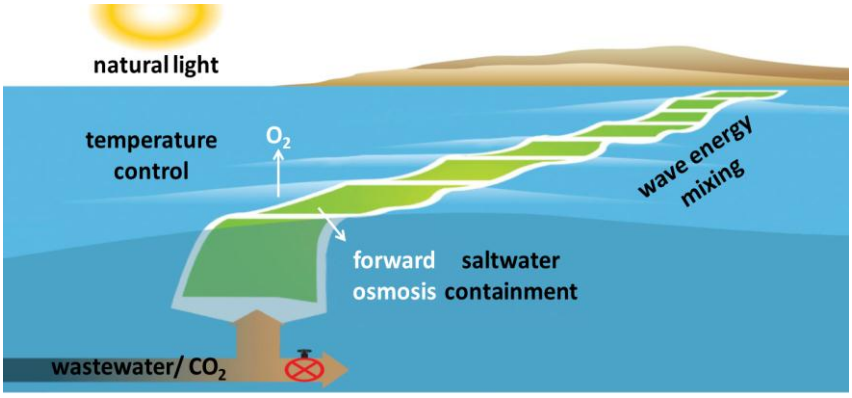


Figure 1.10: Schematic of the Offshore Membrane Enclosure for Growing Algae (OMEGA). This system is an excellent example of creative low energy solutions to a variety of problems associated with microalgal culturing. Adapted from Pulz *et al.* (2009).

Based on current technology alone, it is not feasible to grow microalgae solely for biodiesel production. It needs to be more economically viable with a positive energy return (Pittman *et al.,* 2011). At present there are many small start-up companies, from which some larger companies will potentially near commercialisation. This could make market entry more difficult for smaller local businesses (Parker 2012). Research seems to suggest that integration of small local plants will be vital to the success of algal biodiesel, in which case it will become important for them to be supported (Wagner, 2007), depending on availability and suitability of land (Parmar *et al.*, 2011).

1.3.3 Legislation

Several external factors such as oil prices, stricter GHG emission targets and carbon trading schemes (currently ~$200t CO_2) will also affect the future viability of algal biodiesel (Stephens *et al.,* 2010). Devices such as tax credits or exemptions could incentivize and greatly influence the development of particular technologies (Carriquiry *et al.*, 2011). Carbon tax (an economic device for incentivising the reduction of carbon dioxide emissions from fossil fuel sources) can promote the use of biofuels. If carbon tax on fossil fuels as used to subsidy biofuel costs it could significantly aid the penetration of biofuels into the energy sector (Timilsina *et al.*, 2011).

Governments could also help with underfunding from the private sector in order to deploy certain technologies. Investments part financed by the US Department of Energy have been very effective at reducing the cost of enzymatic digestion of cellulosic ethanol biofuels.

Biodiesel still more expensive than regular diesel and therefore will need to remain subsidized (Razon and Tan, 2011).

Policies regarding the way in which biofuels are produced, consumed and traded are fast changing (Carriquiry *et al*., 2011). Previous legislation is often modified and now many countries have policies to incentivize supply and use of biofuels regardless of the environmental costs of production or relative benefits they provide (i.e. net energy ratio) (Carriquiry *et al*., 2011). There are some exceptions notably in the US and EU. For example tax credits are preferentially given to second generation biofuels than first generation (Carriquiry *et al*., 2011). It is important to consider trends in regional and international development to exploit synergies and maximize benefits (e.g. biofuels that do not match international standards would not progress beyond a domestic market) (Carriquiry *et al*., 2011).

An important question to ask when considering investment into microalgal biofuels is; what is the priority for cost reduction? For cellulosic ethanol the major cost is conversion, yet for microalgae capital cost of plant development is often highest (Leh and Posten, 2009). Given current research status and uncertainties of any major developments what would help make algal biofuels cost-viable, would be to give different support depending on nature of the production process (Parker 2012). In particular an integrated approach combining rural development, climate change, and energy provision could prove very valuable when considering biodiesel production from microalgae (Carriquiry *et al*., 2011). There are likely to be method preferences for different locations (certain growth methods will vary in different geographical regions) and local legislation should reflect this (Shirvani *et al.*, 2011).

1.4 Aims and objectives

The project began with the purpose of improving algal strains for the chemical conversion of CO_2, with the intention of initiating and developing algal research at the University of Bath. Broad aims included; investigating methods to lower the energetic cost of microalgal product extraction and the isolation of novel strains from environmental samples. Objectives remained flexible, due to the infancy of algal research at Bath.

Objectives included:

- Method development of basic laboratory techniques for maintaining and culturing microalgal stocks, isolation of algae from environmental samples, mutagenesis, DNA extraction and molecular identification of microalgae.
- The size and strength of microalgal cells, renders many lipid extraction methods inefficient, energy intensive and expensive. Characterisation of the cell wall with the intention of simplifying or de-energising product extraction was investigated using enzymatic digestion, staining, sonication and electron microscopy.
- The genetic modification of microalgae is believed to have the largest impact in their development as a commercial organism. Mutagenesis and screening for the generation and isolation of novel strains underwent development using the oleaginaceous model alga *C. emersonii*.
- Microalgae are incredibly diverse group of organisms. However only a select few species are studied and used commercially. Bioprospecting is believed to become an invaluable tool for the development of algae-based bioresources, in particular species isolated from extreme environments. The city of Bath is home to one of the only 'hot' springs in the UK, the Roman Baths. This unique natural habitat was the focus for isolation and identification of novel thermo-tolerant microalgal strains.
- Interesting or unusual microalgal species may behave entirely differently when under certain culture conditions. The temperature tolerance, lipid quantity and profile of microalgal isolates from the Roman Baths were investigated.
- Solvent extraction is the standard method for lipid extraction from microalgal samples, however it is a time consuming process delaying data analysis. Various laboratory lipid extraction methods were also investigated in an attempt to accelerate experimental analysis.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Unless otherwise stated, all chemicals were sourced from Sigma Aldrich.

0.1M sodium phosphate buffer (pH8) was prepared as described by Hayat (1986). 4ml 0.2M monobasic stock (13.9g NaH_2PO_4 in 500ml dH_2O) and 71ml 0.2M dibasic stock (28.4g Na_2HPO_4 in 1l dH_2O) were combined and enough dH_2O added to make 600 ml of final solution. Solutions were then autoclaved.

2.1.2 Algal species

Original stocks of *Chlorella vulgaris*, *Chlorella emersonii* and *Scenedesmus vacuolatus* (labeled VT-1 until identified at University of Bath) were obtained from the University of the West of England. *Haematococcus pluvialis* was sourced from the Culture Collection of Algae and Protozoa (CCAP) Oban, Scotland. *Pseudochoricystis ellipsoidea* 'Obi' and 'Ni' were donated by the DENSO Corporation. *Coelastrella saipanensis*, *Choococcidiopsis thermalis*, *Hantzschia* sp., *Klebsormidium* sp., *Microcoleus chthonoplastes*, *Mistogocladus laminosus* and *Oscillatoria sancta* were isolated from the Roman Baths and identified at the University of Bath as described in Chapter 5. These strains were deposited at the CCAP on the 15.05.12 and were assigned the following accession numbers; *Coelastrella saipanensis* (CCAP 217/9), *Choococcidiopsis thermalis* (CCAP 1423/1), *Hantzschia* sp. (CCAP 1030/1), *Klebsormidium* sp. (CCAP 335/20), *Microcoleus chthonoplastes* (CCAP 1449/2), *Mistogocladus laminosus* (CCAP 1447/9) and *Oscillatoria sancta* (CCAP 1459/46).

Freezing of microalgal stock cultures is not suitable for routine subculturing as reactivation of cells needs long periods of incubation for recovery (Andersen, 2005). Work described by Malik (1995) has shown microalgal stock cultures to remain viable and stable for over 12 month in suitable media in wide mouth Erlenmeyer flasks under low light and temperatures (specific to individual strain). No specialist equipment or subculturing is required and recultivation did not necessitate time for cell recovery. This method was employed for maintaining our stock cultures. Algal stocks were maintained by cultivating in 50ml skirted centrifuge tubes (Greiner Falcon) in preferred medium (with monthly manual shaking) and on agar media plates (preferred medium with 1% (w/v) agar) stored on a lab bench (~20ºC,

~50µMol m^{-2} s^{-1}). To increase cell survival and stability, various adsorption materials (e.g. activated charcoal) or stabilisers may be added, but were not over the period of this project (Malik, 2005).

C. emersonii was used as the 'standard' test organism for mutagenesis, culturing, cell wall and extraction methods studies. This is due to its high oil content (63wt%, Scragg *et al.*, 2002), and larger size than *C. vulgaris*, making it easier to visualise under the light microscope in order to assess changes to its morphology and responses to cellular staining.

2.1.3 Algal culture media

All green eukaryotic algae (*C. vulgaris, C. emersonii, S. vacuolatus, H.pluvialis, P. ellipsoidea, C. saipanensis, K. sp.*) were cultured in Bolds basal medium (BBM) prepared as follows.

To 700-800ml of distilled water, 10ml of each of the six 'main' stock solutions (Table 2.1) and 1ml of each of the four 'micronutrient' stock solutions (Table 2.2) were added and made up to 1l with dH_2O. Final pH adjusted to 6.6 with HCl acid NaOH. Solutions were autoclaved.

Table 2.1: Main stock solutions for the preparation of Bolds basal medium.
From MacDonald (2008).

Stocks	Formula	Name	Conc. (g l^{-1})	Conc. (Molar)
1	$NaNO_3$	Sodium nitrate	25.0	2.94×10^{-3}
2	$CaCl_2.2H_20$	Calcium chloride	2.5	1.7×10^{-4}
3	$MgSO_4.7H_2O$	Magnesium sulphate	7.5	3.04×10^{-4}
4	K_2HPO_4	dipotassium hydrogen orthophosphate	7.5	4.31×10^{-4}
5	KH_2PO_4	potassium dihydrogen orthophosphate	17.5	1.29×10^{-3}
6	NaCl	sodium chloride	2.5	4.28×10^{-4}

Table 2.2: Micronutrient stock solutions for the preparation of Bolds basal medium.
From MacDonald (2008).

Stocks	Formula	Name	Conc.	Conc. (Molar)
1	H_3BO_3	Boric acid	11.42 g l^{-1}	4.62×10^{-4}
2	H_2SO_4	Sulphuric acid	2.5ml l^{-1}	4.48×10^{-5}
	$FeSO_4.7H_2O$	Iron (II) sulphate	12.45 g l^{-1}	4.48×10^{-5}
3	Na_2EDTA	Sodium EDTA (anhydrous)	50 g l^{-1}	4.42×10^{-4}
4	$ZnSO_4$	Zinc sulphate	8.82 g l^{-1}	7.67×10^{-5}
	$MnCl_2$	Manganese Chloride	1.44 g l^{-1}	1.82×10^{-5}
	MoO_3	Molybdenum (VI) oxide	0.71 g l^{-1}	1.23×10^{-5}
	$CuSO_4$	Copper (II) sulphate	1.57 g l^{-1}	1.57×10^{-5}
	$Co(NO_3)_2$	Cobalt (II) nitrate	0.49 g l^{-1}	4.21×10^{-6}

The diatom *Hantzschia* sp., was cultured in modified BBM medium, and named 'diatom medium' (DM) modified as follows. To 1l BBM 1ml $Na_2O(SiO_2)x.xH_2O$ and 1ml of 'vitamin stock solution' added ('vitamin stock solution' of 200ml contained: 0.008g cyanocobalamin, 0.008g thiamine, 0.008g biotin) (adapted from Barsanti and Gualtieri).

All cyanobacteria ('blue-green algae') were cultured in Blue-Green medium (BG-11) prepared as follows (adapted from Barsanti and Gualtieri, 2006). To 100ml of stock solution 1, 10ml of stock solutions 2-8 and 1ml stock solution 9 was added (Table 2.3). Mixture was made up to 1l with distilled water pH adjusted to 7.1 with 1M NaOH or HCl and autoclaved.

Table 2.3: Stock solutions for preparation of Blue-Green 11 medium.
Adapted from Barsanti and Gualtieri (2006).

Stocks	Formula	Conc. (g l^{-1})
1	$NaNO_3$	15
2	K_2HPO_4	4
3	$MgSO_4$	3.7
4	$CaCl_2$	2.7
5	Citric acid	0.6
6	Ammonium ferric citrate	0.6
7	$EDTANa_2.2H_2O$	0.1
8	Na_2CO_3	2
9	H_3BO_3	2.86
	$MnCl_2$	1.15
	$ZnSO_4.7H_2O$	0.22
	$Na_2MoO_4.2H_2O$	0.39
	$CuSO_4.5H_2O$	0.08
	$CoCl_2.6H_2O$	0.02

Where algae were cultured on agar plates, plates were prepared by adding 1% (w/v) of agar to preferred media. After autoclaving molten agar-media was poured into 90mm Petri dishes (Sterilin). Unless otherwise stated liquid cultures were cultivated in 100ml of preferred media in a 250ml conical flask, stoppered with a foam bung and covered in aluminium foil. These were autoclaved prior to inoculation.

BBM with added sodium acetate (0.1% w/v) was also used in some experiments as a carbon source and referred to as 'BBM C+'. Acetate was chosen as it is a common microbial carbon source and glucose is often toxic to microalgae (Perez-Garcia *et al.*, 2011). For nitrogen starvation of algae (which often leads to accumulation of high levels of intracellular lipids), sodium nitrate (main stock solution 1) was omitted but BBM otherwise prepared as normal. This method for creating nitrogen free media is also applied to the aforementioned DM and

BG-11 media for liquid and agar plates. A graph illustrating the differences in nutrient concentration between BBM and BG-11 is given in Appendix A.

2.1.4 Growth equipment

Algal growth was carried out in a SANYO MLR-351 'environmental test chamber' at 100 xg with a SANYO orbital shaker, at 25ºC, with 150-200 µMol m^{-2} s^{-1} of light on a 12 h on, 12 h off cycle. Truly synchronised cultures are a prerequisite for good experimental sensitivity with regards to the cell cycle. Good synchonity may need close to 12:12h light: dark cycles (Otero and Goto, 2005). Two SANYO MIR S100 shaker platforms (capable of holding six 250ml conical flasks) were fixed to shelves inside the chamber (Figure 2.1). Light measurements taken with a LI-COR 250A light meter.

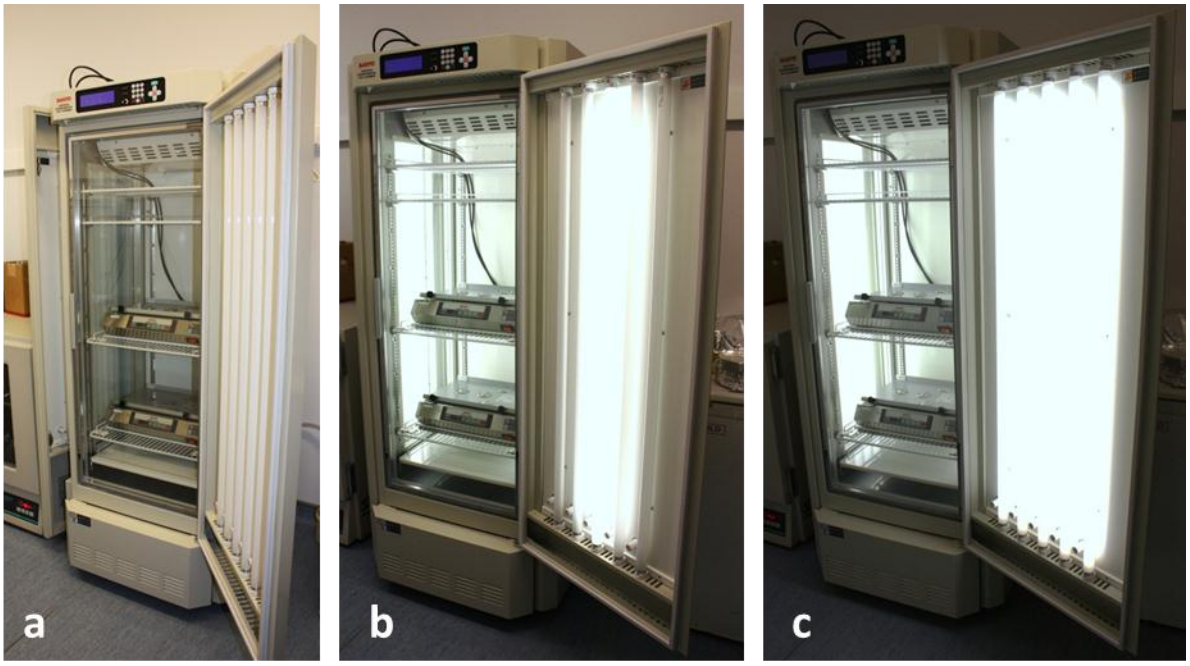


Figure 2.1: The SANYO MLR-351 'environmental test chamber'.
(a) lights off, (b) light setting 3, (c) light setting 5.

The lights in the SANYO test chamber consist of 6 settings (off, 1,2,3,4,5) and is illuminated on 3 sides. As a result, different flask positions in the test chamber received slightly different quantities of light (Figure 2.2). This difference does not become marked until the higher light settings of 4 and 5.

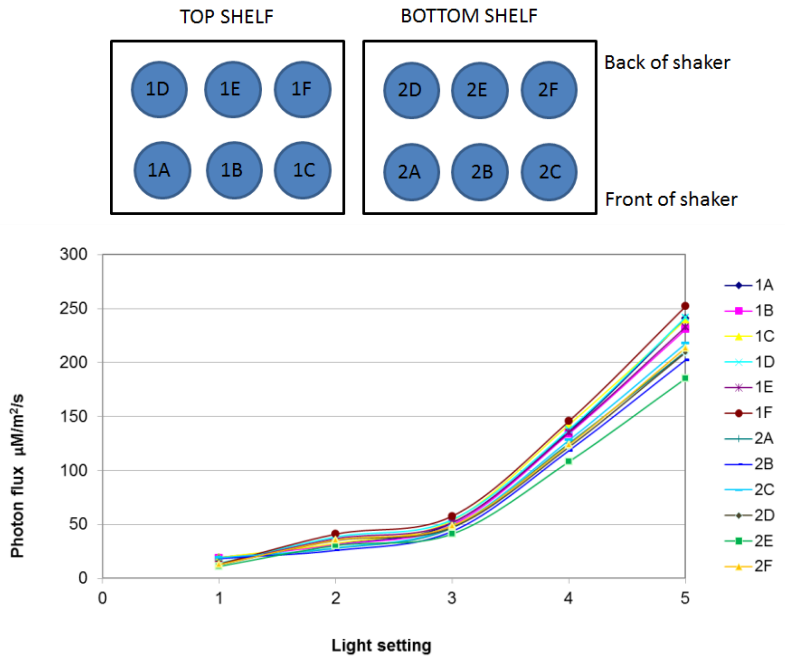


Figure 2.2: Light levels at different positions within the environmental chamber.
(Above) 'map' of the 250ml flask positions within the SANYO test chamber. (Below) graph to show differences in photon flux between different flask positions under different light settings.

For a few experiments which required larger quantities of biomass (>1g dry microalgal biomass) for which light was not a critical factor (e.g. extraction method experiments, Chapter 7) *C. emersonii* was cultivated in 8l 'ExAlga' vertical photobioreactors (vPBR, Figure 2.3) in a Fitotron ® plant growth room (WEISS Gallenkamp).

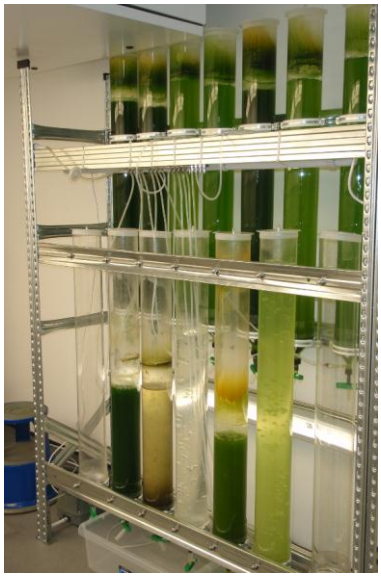


Figure 2.3: The 8l 'ExAlga' vertical photobioreactors (vPBR) in the Fitotron ® plant growth room.
These vPBRs were used for the production of larger quantities of biomass of *C. emersonii*.

2.1.5 Freeze drying (lyophilisation)

Small algal samples for dry weight measurements were pelleted in 2ml Eppendorf tubes at 13,000xg for 5 min in an 'Eppendorf 5415C' centrifuge and supernatant removed. This was carried out 1ml at a time, 3 times in total. For 100ml cultures, half of each culture was centrifuged in 50ml skirted centrifuge tubes (Greiner Falcon) in an 'Eppendorf 5810R' centrifuge (with an Eppendorf A-4-62 rotor) at 3000xg for 5min, supernatant removed and remaining culture added to the tube and centrifuged again. For very large sample volumes of 8l, 2l of culture was centrifuged in 500ml centrifuge pots in a Beckman Coulter Avanti J-25 Centrifuge (equipped with a Beckman JLA rotor) at 8000xg for 10 min and supernatant removed. For these large volume samples, some dH_2O was added back into the pellets to form a thick slurry so it could be pelleted prior to lyophilisation by dropping the algal slurry with a plastic Pasteur pipette into $N_2(l)$ and sieved into a 50ml falcon tube. This formed frozen algal beads for more even lyophilisation.

Samples in 2ml Eppendorfs were lyophilised using a Savant Speed Vac Concentrator connected to an Edwards Modulyo FreezeDryer with an Edwards oil mist filter EMF10. Large volume samples in 50ml skirted falcon tubes were sealed with a layer of parafilm and using a needle, small holes made in the film before placing under a vacuumed 'bell jar' on top of the aforementioned freeze dryer. Samples were then freeze dried over 24h (2ml samples) or 48-56h (larger samples).

2.1.6 Imaging and microscopy

Photographs of plates, flasks and other equipment were taken with a Canon Rebel XSi 450D DSLR camera. Images of cells in a haemocytometer and plate colonies were taken using a Nikon eclipse TE2000-5 inverted microscope.

Slide preparations were viewed under a Nikon Eclipse 90i microscope. Images were taken with Nikon Digital Sight DS-U1 colour camera for light microscopy, and a D-Eclipse C1 for confocal images. Images were processed using Adobe Photoshop Elements.

For viewing of fluorescent stains calcofluor and DAPI under the Nikon Eclipse 90i microscope, ultraviolet (UV) excitation wavelength of ~360nm were used. For viewing of nile red and BODIPY stains excitiation wavelength of ~485nm were used for viewing with the confocal.

2.1.7 Primer design

Primers were designed using certain criteria (Table 2.4) and the target sequence. Sequences were checked using NetPrimer (Premier Biosoft International) against reverse primers to be used and for melting temperatures.

Table 2.4: Guidelines used for primer design.
From Tiwari (2009).

Ideal features	Features to avoid
Primer length of 23-25bp	Hairpins, dimers, cross-dimers
Annealing temperature of 56-60°C	Many similar nucleotides together (e.g. CCCCC)
GC content of 25-40%	5' overhang is acceptable
Fragment size of800-900bp	
A and T at 5' end	
C and G at 3' end	
NetPrimer rating 90-100%	

Two pairs of initial primers were used for the amplification of cyanobacterial 16S (modified from Taton *et al.*, 2003) and eukaryotic 18S (Cuvelier *et al.*, 2008) rDNA sequences (Table 2.5). Due to the nature of Sanger sequencing, a maximum of 500-700bp of the ~1.7kbp fragments could be sequenced at any one time. For some samples this required designing of 'secondary primers' (#2) from sequences received.

Table 2.5: Primer pairs used for the amplification of 16S (cyanobacteria) or 18S (eukaryotic algae) rDNA gene regions.
Second primer sets were created if sequence read-lengths were <1000bp.

Species	1st primer pair (name/Tm)	2nd primer pair (name/Tm)
Cv	ACCTGGTTGATCCTGCCAG (U18SF/57.7°C)**	ATGGTGGTAACGGGTGACG (U18SFCV#2/59°C)
	TGATCCTTCTGCAGGTTCAC (U18SR/55.4°C)**	U18SR
Ce	ACCTGGTTGATCCTGCCAG (U18SF/57.7°C)**	ATTGGAGGGCAAGTCTGG (U18SFCE#2/55°C)
	TGATCCTTCTGCAGGTTCAC (U18SR/55.4°C)**	U18SR
Sv	ACCTGGTTGATCCTGCCAG (U18SF/57.7°C)**	TTCTTAGAGGGACTATTGGCG (U18SFVT1#3/57°C)
	TGATCCTTCTGCAGGTTCAC (U18SR/55.4°C)**	U18SR
Os	AGAGTTTGATCCTGGCTCAG (U16SF/54.0°C)*	U16SF
	ACGGCTACCTTGTTACGACTT (U16SR/56.2°C)*	TATCTAATCCCATTCGCTCC (GBU16SR#2/54.6°C)
Mc	AGAGTTTGATCCTGGCTCAG (U16SF/54.0°C)*	U16SF
	ACGGCTACCTTGTTACGACTT (U16SR/56.2°C)*	AACCACATACTCCACCGC (KBU16SR#2/53.1°C)
Ml	AGAGTTTGATCCTGGCTCAG (U16SF/54.0°C)*	-
	ACGGCTACCTTGTTACGACTT (U16SR/56.2°C)*	
K sp.	ACCTGGTTGATCCTGCCAG (U18SF/57.7°C)**	-
	TGATCCTTCTGCAGGTTCAC (U18SR/55.4°C)**	
Cs	ACCTGGTTGATCCTGCCAG (U18SF/57.7°C)**	ATTACCCAATCCTGATACGG (RBSU18SF~2/54.2°C)
	TGATCCTTCTGCAGGTTCAC (U18SR/55.4°C)**	U18SR
Ct	AGAGTTTGATCCTGGCTCAG (U16SF/54.0°C)*	-
	ACGGCTACCTTGTTACGACTT (U16SR/56.2°C)*	
H sp.	ACCTGGTTGATCCTGCCAG (U18SF/57.7°C)**	ATCATTCAAGTTTCTGCCC (RBDU18SF#2/52.1°C)
	TGATCCTTCTGCAGGTTCAC (U18SR/55.4°C)**	U18SR

* (modified from Taton *et al.*, 2003), **(Cuvelier *et al.*, 2008).

2.1.8 Sequencing services and analysis software

Sequencing was carried out by Source BioScience. Received sequences were viewed using Sequencher and ambiguous nucleotides confirmed where possible and changes recorded. Sequences were assembled using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA).

2.1.9 Nuclear magnetic resonance imaging (NMR)

H^1NMR was carried out using a Bruker 500 NMR spectrophotometer. Samples were dissolved in deuterated $CHCl_3$, with added benzaldehyde as an internal standard (Table 2.6)

Table 2.6: Quantities of benzaldehyde added as an internal standard to samples analysed by H^1NMR.

Sample name	Benzaldehyde (g)
Soxhlet 1	0.005
Beadbeater 1	0.034
Sonication 1	0.035
Microwave 1.1	0.022
Microwave 2.1	0.043
AntonPaar 1.3	0.007
AntonPaar 2.3	0.047

2.1.10 Fourier transform infrared spectroscopy (FT-IR)

FTIR carried out using Perkin Elmer Precisely Spectrum 100 FTIR spectrometer and Universal ATR sampling accessory. Spectra analysed using PerkinElmer Spectrum Express.

2.1.11 Data processing and statistics

All numerical data and graph for growth and GC-MS data was processed in was carried out in Microsoft Excel (Office 2010). Standard deviation applicable to growth measurements and some GC-MS data was also manipulated in Excel for the application of error bars to graphs and generation of any GC-MS traces.

2.2 Methods

2.2.1 Measuring growth by cell count

Measuring cell count was achieved using an Improved Neubauer haemocytometer (unless otherwise indicated). For densities of up to ~2.5 x10^6 cells ml^{-1}, all grid squares were counted. For densities over 2.5 x10^6 cells ml^{-1} 5 grid squares were counted (central and four outermost corners), this is common practice for high cell densities. In order to have an accurate measure of error using these methods, the standard deviation was calculated for each method using *C. emersonii* and 5 readings per method (Table 2.7). The Table illustrates the change in error between using different methods at different concentrations.

Table 2.7: Standard deviations when using a haemocytometer for calculating cell concentration.
Five cell count readings taken using various cell concentrations of *C. emersonii* to compare standard deviation values to calculate error based on method and concentration.

Mean concentration x10^6cells ml^{-1}	Method	Standard deviation
18.420	5 grid count	0.266
8.900	5 grid count	0.345
5.090	5 grid count	0.303
4.654	All	0.488
2.529	5 grid count	0.208
2.528	All	0.653

2.2.2 Measuring growth by spectrophotometer

It has been shown that dry weight correlates strongly with a species-specific pre-calibrated spectrophotometric optical density (OD) curve (Griffiths *et al.*, 2011). For some experiments this was used as a growth measurement alongside cell count. OD was measured using Spectronic Unicam 'Heλios γ' spectrophotometer at λ550nm, by first inserting a blank (dH_2O) to calibrate the system before measuring samples.

2.2.3 Measuring growth by mass

Dry weight measurements for growth were recorded by lyophilising samples of culture in preweighed 2ml Eppendorf tubes (described in Chapter 2.1.5) which were reweighed after freeze drying. Sample mass calculated to the nearest 0.0001g using a Sartorius analytic A200S mass balance.

2.2.4 Measuring growth by flow cytometer

Where indicated growth has been measured by cell count using Millipore Guava-Easycyte ™, using software 'InCyte' GuavaSoft 2.2.2. For cell densities above ~$1.0x10^6$cells ml^{-1} cells were diluted 1/100 by adding dH_2O.

2.2.5 Sample staining and microscopy preparation protocols

A total of 8 stains were used for staining all algal stock species, each requiring a specific sample preparation protocol (Table 2.8). Samples were subsequently transferred to a glass microscope slide with a coverslip for viewing under the light microscope or confocal.

Table 2.8: Summarised protocols for a variety of staining methods applied to algal strains.

Stain	Procedure	Stain target
Aceto orcein	Samples were pelleted* and stained in aceto orcien (1g orcein in 45ml glacial acetic acid) for 10min (Scott, 2008).	Nucleic acids
BODIPY (Invitrogen)	25µl of DMSO and 1µl 1% (w/v) BODIPY (in acetone) were added to 100µl of cell culture. This was mixed well and stained for 1min in the dark. Prior to viewing samples were diluted by adding 1ml of dH_2O (adapted from Satoh *et al.,* 2010).	Lipids
Calcofluor	Samples were pelleted* and supernatant removed and replaced with and equal volume of 0.05% w/v calcofluor white ($C_{40}H_{42}N_{12}O_{10}S_2.2Na$) in acetone. This was then incubated in the dark for 10min. Samples were then pelleted again and washed twice in 25mM phosphate buffer (pH8) and finally resuspended in 100 µl buffer (Honjoh *et al.,* 2003).	β1,4 and β1,3 glucans (non-specific cell wall stain)
Coomassie blue	To 500µl of cell culture 500µl Coomassie blue stain was added (200mg coomassie blue in 600ml ethanol:dH_2O:methanol 1:4:25) and was left to stain overnight. Cells were then pelleted* and washed with dH_2O before resuspending in 25mM phosphate buffer (pH8) (Qi, 2009)	Protein
Crystal violet	To 1ml of pelleted* cell culture 1ml of 0.2% crystal violet (filtered) was added and samples left to stain overnight. Cells were pelleted and excess stain removed and cells resuspended in dH_2O (adapted from Burczyk, 2009).	Peptidoglycans (can be used for nucleic acid staining)
DAPI	To 20µl cell culture, 1µl DAPI and 5 µl wetting agent (2% v/v Triton X) was added and left to stain for 1h (Doughty, 2010).	Nucleic acids (A-T rich regions)
Nile Red	25µl of DMSO and 1µl 1% (w/v) nile red in (acetone) were added to 100µl of cell culture. This was mixed well and stained for 1min in the dark. Prior to viewing samples were diluted by adding 1ml of dH_2O (adapted from Satoh *et al.,* 2010).	Lipids
Sudan black	Sample was pelleted and resuspended in sudan black 0.1% (w/v) (in 70% ethanol) for 8 min. Sample was then washed twice in phosphate buffer (pH8) over 10 min (adapted from Rodríguez-García *et al.,* 2003).	Lipids

*Pelleted = 12,000xg for 3 min in an Eppendorf 5415C centrifuge.

Outlined as follows, is the procedure for preparation of diatom silica 'skeletons' for viewing by light microscopy. An obtained pellet of cells was washed with dH_2O twice and centrifuged at 3000xg over 10 min. Supernatant was removed and pellet resuspended in 1ml hydrogen peroxide (30% w/v) and mixed well. Tube then placed in a water bath at 80-90°C for 1h and then allowed to cool. Sample was transferred to 15ml falcon tube with ~10ml dH_2O, mixed and centrifuged at 3000xg for 3min. Supernatant then removed and pellet washed twice with distilled water, centrifuging each time and removing supernatant. Working in a clean flow hood 0.5 ml of mixed suspension was dropped onto coverslips and dried using a hotplate at ~50°C. 1 drop of DPX mounting medium was dropped onto glass slides and using forceps the coverslips with dried diatom were inverted and placed over the drops of DPX. Assembled slides were then dried on the hotplate (at 80°C) overnight. Adapted protocol (Spaulding, 2011) replaced Naphrax with DPX as a mounting medium.

2.2.6 Scanning electron microscopy (SEM)

To 25ml of double strength cell culture, fixative was added (5ml 25% GDA) and made up to 50ml with filtered dH_2O (resulting in 2.5% GDA in 'normal' strength culture medium). To this 0.5g potassium ferrocyanide was added and mixed well. Thermanox coverslips were placed in a small glass Petri dish. The wells were half filled with algal culture and left at room temperature for 1h. Very slowly culture medium was pipetted off at the side of the wells, away from coverslips so as not to disturb cells. Fixative was pipetted into wells slowly, keeping at the edge of the well. Samples were left in fixative overnight.

Postfixation was carried out using aqueous 1% osmium tetroxide for 1h in fume hood at room temperature. Sample then washed in dH_2O twice over 10min.. Sample then stained in 2% aqueous uranyl acetate for 1h in the dark. Samples were then dehydrated in increasing concentrations of acetone (twice over 10min at each concentration), 50, 70, 90, 100%. Samples then placed in 1:1 acetone:HMDS for 15min and then in 100% HMDS x2 over 10min. Samples were kept under liquid during all stages. As much HMDS was pipetted off as possible and sample dish was left in fume hood to evaporate remaining HMDS over 1-2h. Samples then cut and mounted onto conductive carbon adhesive tabs before undergoing gold sputtering using an Edwards Sputter Coater S150B.

Scanning electron microscope images were taken using JEOL JSM6480LV and Oxford INCA x-ray analyser used for mapping, scanning and quantitative analysis.

2.2.7 Transmission electron microscopy (TEM)

Algal samples for TEM were cultured as described in Chapter 2.2.27. After 10 days, half of each culture underwent nitrogen starvation. Approximately 5ml of each algal culture was centrifuged and culture medium replaced with 0.1M sodium cacodylate buffer pH 7.4 (2.14g of sodium cacodylate in 40ml of dH_2O pH adjusted to 7.4 using HCl, made up to 50ml with dH_2O) and mixed well. Cells were then pelleted (12,000xg, 5min in an Eppendorf 5415C centrifuge), wash solution removed and resuspended in fixative solution (2.5% gluteraldehyde, 2% formaldehyde, 0.5% acrolein (Agar Scientific) in 0.1M sodium cacodylate buffer pH 7.4) and left overnight at 4ºC.

Cells subsequently pelleted, fixative removed and replaced with 0.1M sodium cacodylate buffer and washed three times over 20min. The last wash solution was removed and replaced with aqueous 1% osmium tetroxide and 1% potassium ferrocyanide for 1h in fume hood at room temperature. Meanwhile 3% low melting point agarose in distilled water was prepared in a glass test tube and kept in water bath at 35ºC. Cells were then pelleted and washed in dH_2O three times over 15min. Cells pelleted again, supernatant removed and pellet transferred to a water bath at 35ºC. Cells were then encapsulated in warm agarose, stirred with a cocktail stick and droppered onto cool glass slides to solidify. 0.5mm sections of the agar drops were cut and transferred to glass vials and stained in 2% aqueous uranyl acetate, for 1h in the dark.

Samples were dehydrated in increasing concentrations of acetone (twice over 15min at each concentration), 30, 50, 70, 95%. Samples then placed in 100% dry acetone, changing 4 times over 30min at room temperature. After dehydration samples infiltrated with TAAB low viscosity resin and 100% dry acetone (1:1) for 2h. Liquid mixture then removed and replaced with 100% resin and put under vacuum for 1h (or until acetone removed). Final liquid mixture removed and replaced with fresh resin and left overnight.

Samples then placed in moulds with fresh resin and polymerised in an oven at 70ºC for 24h. Samples were sectioned (~0.1µm thick) using a Reichert-Jung 'Ultracut E' Microtome, using a Diatome diamond knife (Knife number MS11872). Sections were supported on SIRA 300 mesh copper grids.

Transmission Electron Microscopy (TEM) images were taken at Johnson Matthey (Sonning Common site) using FEI Technai F20 Field emission gun transmission electron microscope

(operating voltage 200kV). Due to the high voltage used, samples were carbon coated prior to viewing using an Emitech K950X carbon coater (100ms) and carbon rods. Some samples were viewed using the facilities at the University of Bath (Transmission electron microscope JEOL JEM1200EXII (operating voltage of 120 kV) and images captured with Gatan Dualvision digital camera), these are indicated in the text.

2.2.8 Enzyme treatment

Formation of protoplasts (or changes to the cell wall) was calculated using two methods, the first adapted from Hatano *et al.* (1992). 2ml of log phase culture medium (~$1x10^6$ cells ml^{-1}) was centrifuged and supernatant removed. To this 2ml of 25mM sodium phosphate buffer with 0.5M mannitol was added. A 12% w/v solution of enzyme in 25mM sodium phosphate buffer was made, homogenized and filtered through a 0.2µm syringe filter. The algal preparation was added to the enzyme preparation (1:5). The mixture was then incubated in the dark at 25^{o}C, 50xg, for 3h.

To analyse samples 2µl of the mixture was transferred to a microwell and 6µl of 2% SDS solution was added. After 1 minute any 'protoplasts' formed sank to the bottom of the well with unaffected cells. Microwells were then viewed with an inverted microscope at 10x magnification. These volumes typically gave a monolayer of cells. An image of a ¼ well was taken and a grid overlaid (Figure 2.4). This allowed affected cells to be estimated as 'protoplasts' had an affinity for well edges. The protocol was modified from Yamada and Sakaguchi (1981; 1982).

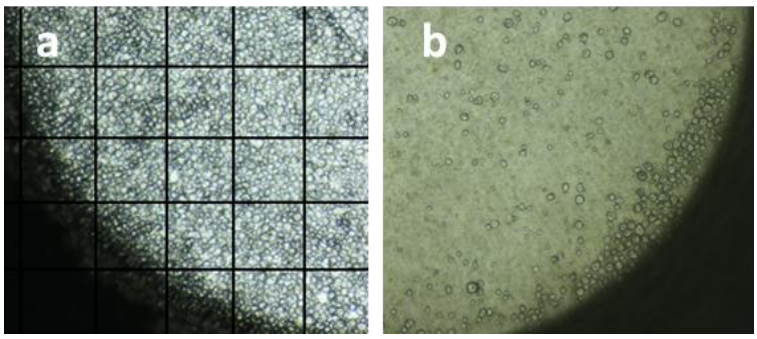


Figure 2.4: Image taken of a ¼ microwell or counting algal protoplasts.
(a) the grid overlay in order to ease cell counting and (b) larger cells with an affinity for the edges of microwells.

The second method for analysing the effect of enzymes on the microalgal cell wall was based on a method by Honjoh *et al.*, (2003). The algal enzyme suspension was pelleted and

resuspended in 0.05% calcofluor in 25mM sodium phosphate buffer containing 0.5M mannitol and stained in the dark for 10min. Cells were then washed 4 times with the aforementioned buffer and observed using the fluorescence microscope (excitation 330-380nm, emission >420nm).

Enzymes tested included cellulose, hemicellulase, pectinase, chitinase, achromopeptidase, 'driselase' (containing laminarinase, xylanase and cellulase), β-1,3-glucanase, ligninase, laminarinase, xylase and 'macerozyme R-10' (containing cellulose, hemicellulose and pectinase). A suspected mixture of 'callase' enzymes were also sourced from tobacco buds and tested, the extraction of this enzyme is described below.

Prior to meiosis microsporocytes in angiosperms synthesise a specialized cell wall comprised of callose. Upon completion of meiosis callose is rapidly digested by callase (usually comprised of secreted exo and endo β-1,3-glucanases – a potent enzyme cocktail) and microspores are released (Worral *et al.*, 1992) and (Hird *et al.*, 1992). Buds of ~7mm (*Nicotiana* sp.) or ~24mm (*Lilium longiflorum*) were removed and a slice of anther from each bud squashed with 5μM aceto-orcein stain under a cover slip and viewed to determine stage of microsporogenesis (Tanaka, 1991). Buds which showed intact tetrads and liberated microspores had all anthers removed and ground with ~500μl of 25mM sodium phosphate buffer. This enzyme solution was then used to treat algae as described above.

2.2.9 Extraction of 'algaenan'

The following method is adapted from Allard *et al.,* (1998). A large quantity of lyophilised algal biomass (over 10g) was put under reflux in a 500ml round bottom flask with $CHCl_3$:MeOH (2:1 v/v) for 3h, the solid filtered and rinsed with $CHCl_3$ (to extract lipids). The solid was then hydrolysed in 2N TFA (100ºC, 3h), then further hydrolysed in 4N TFA (100ºC, 18h) before final hydrolysis in 6N TFA (100ºC, 18h) (to extract polysaccharides). Solid residue was thoroughly washed with dH_2O before putting under reflux in dH_2O for 1h. Solid then refluxed in $CHCl_3$:MeOH (2:1 v/v) for 3h, saponified by reflux in 5% KOH in 2-methoxyethanol:H_2O (88:12 v/v) for 1h. Solid then put under reflux in 6N HCl 100ºC for 24h. Solid residue filtered and washed with H_2O before refluxing in H_2O for 1h and finally refluxing $CHCl_3$:MeOH (2:1 v/v) for 3h. Final residue was filtered out and rinsed with H_2O before allowing to dry at room temperature overnight.

2.2.10 Cultivation of Fusarium oxysporum f.sp elaeidis and exposure to algaenan

Fusarium oxysporum f.sp. *elaeidis,* an oil palm pathogen, was donated by Hefni Rusli (University of Bath) for investigating the digestion of algaenan with a live fungus. Czapek-Dox media with different concentrations of sucrose (carbon source) were prepared (Table 2.9). Minimal carbon media (Cmin) was tested alongside carbon deficient media (C-) as some fungi require 'metabolic momentum' in order to synthesise enzymes for the digestion of tough materials (Cooper, 2012).

Table 2.9: Recipe for Czapek-Dox media for the liquid culture of *F.oxysporum* f.sp. *elaeidis* including media modification for the reduction of carbon content.
Adapted from Rusli, (2012).

Component	Formula	Standard Czapek-Dox (C+) g l^{-1}	Minimal sucrose (Cmin) g l^{-1}	Sucrose-free (C-) g l^{-1}
Sucrose	$C_{12}H_{22}O_{11}$	30.000	0.500	0.000
Sodium nitrate	$NaNO_3$	3.000	3.000	3.000
Dipotassium phosphate	K_2HPO_4	1.000	1.000	1.000
Magnesium sulphate	$MgSO_4$	0.500	0.500	0.500
Potassium chloride	KCl	0.500	0.500	0.500
Ferrous sulphate	$FeSO_4$	0.010	0.010	0.010

Liquid media were adjusted to pH 7.3 using HCl prior to autoclaving, as ~pH7 is optimal for cutinase production in *F.oxysporum* (Pio *et al.*, 2008). Due to the formation of a cloudy residue after autoclaving, 15ml of media was filtered using a 25ml NORM-JEX ® syringe and sterile MILLEX GS 0.22µm filter into sterile 100ml conical flasks covered with foil. Flasks were incubated at 28°C at 150xg in a Stuart S150 orbital incubator for 5 days. For the investigation into the digestion of algaenan or algae (autoclaved, frozen and live) by *F.oxysporum* .sp. *elaeidis* flasks were inoculated to give a final concentration of algal cells of ~1.5 x10^6 cells ml^{-1}. For samples containing algaenan a visible quantity was added (not weighable <1mg). The growth of *F.oxysporum* f.sp. *elaeidis* was measured over 5 days using a haemocytometer. In samples where some hyphal growth was observed, 'cell counts' for hyphae were estimated based on the length of an average spore (¼ of the length of the side of the smallest haemocytometer well ~ 0.625µm).

Where *F.oxysporum* sp. *elaeidis* was cultured on agar plates, plates were prepared by adding 1% (w/v) of agar to the media. After autoclaving molten agar-media was poured into 90mm Petri dishes (Sterilin). Plates were inoculated centrally with *F.oxysporum* f.sp. *elaeidis* using 10µl of culture and allowed to dry for 5 min in a laminar flowhood. For the investigation into the digestion of the algal cell wall, 10µl algal samples (autoclaved, frozen and live) were

dropped approximately 1cm from the edge of the plates. In the case of algaenan, small visible samples (~5mm in diameter) were transferred 1cm from the edge of the plate. Plates were incubated at 28°C for 5 days.

After 5 days plates were photographed and viewed under the inverted microscope. Liquid samples containing algae (autoclaved, frozen and live) were transferred to 15ml greiner falcon tubes and pelleted at 3000xg for 5 min. Pellets were then resuspended in 5ml sodium phosphate buffer. 1ml of this sample was then transferred to an Eppendorf tube containing 0.05% calcofluor in 25mM sodium phosphate buffer, inverted to mix, and incubated for 5min. Samples were then transferred to a microscope slide and viewed under the confocal microscope.

Liquid cultures of *F.oxysporum* f.sp. *elaeidis* in 'Cmin' media were transferred to 15ml greiner falcon tube and pelleted (13,000xg, 5min). The supernatant was transferred to a new 15ml greiner falcon tube and a visible quantity of *C. emersonii* algaenan added (<1mg). The sample was then incubated at 28°C, 100xg. After 24hr the sample was filtered and supernatant retained for further processing and GC-MS analysis.

Liquid samples in Cmin media containing algaenan; the control (with no fungus), fungus cultivated in the presence of algaenan and Cmin fungal culture supernatant was subsequently filtered and used for the digestion of algaenan (described above), were all filtered and then subjected to transesterification, washing and GC-MS analysis as follows. To ~15ml filtrate samples 20ml $CHCl_3$:MeOH (2:1) and 1 drop concentrated H_2SO_4 were added into a 50ml round bottomed flask and shaken well. Samples were then transesterified at 80°C and stirred at ~300xg for 3h. Samples were then washed with dH_2O, dried and dissolved in 200µl of dioxane and analysed by GC-MS.

2.2.11 UV mutagenesis

The UV mutagenesis protocol was adapted from MacDonald (2010) and Tripathi *et al.*, (2001). 25ml of algal culture of density $1x10^6$ cells ml^{-1}, was added to a 90mm Petri dish (Sterilin). Sample was mixed by agitating the Petri dish to ensure homogeneity of the solution, from this a 1ml samples were removed into a tube labelled 0 (control). The remaining solution was located directly under a UV biocidal radiation lamp (BioTechne Hepa UV laminar flow hood) for 5 min (~53cm from bulb). After 5 min the solution was agitated, 1ml sample was then taken.This procedure was repeated for samples taken at 10, 15, 20, 25,

30min (this was changed to sampling every 2min after the first UV mutation curves were generated). These samples were then diluted 1 in 100 (10µl samples to 990µl BBM), then further diluted 1 (40µl) in 5 (200µl), to give a final 1 in 500 dilution.

30µl of each sample (and replicates) was then plated onto 90mm Sterilin plates (BBM 1% (w/v) agar) and appropriately labelled. The plates were then sealed with Parafilm and stored in the algal Fitotron ® growth room until colonies appeared. Suitable exposure time for UV mutagenesis was determined by plotting number of survivors against UV exposure.

C. emersonii underwent further mutagenesis with UV. Mutagenesis was carried out as described above at the '10% survival UV mutagenic dose' (for *C. emersonii* this was 11min), omitting the final 1 in 5 dilution step prior to plating. Approximately 100 plates were prepared from this sample in order to screen for mutants.

2.2.12 EMS mutagenesis

Protocol adapted from McCann (2009). 10ml cell culture suspension ($1x10^6$cells ml^{-1}) was centrifuged at 6000xg for 5min and resuspended in 5ml sodium phosphate buffer (pH8). 90µl ethyl methanesulphonate (EMS) added to give a final concentration of 143.25mM (1.8%, w/v). Sample vortexed and incubated for 1h at 30ºC in the dark (to reduce photo-induceable DNA repair mechanisms). 500µl samples were taken at 0, 10, 20, 30, 40, 50, 60 min intervals and mutagenesis quenched by adding 500µl fresh made sterile-filtered 5% (w/v) sodium thiosulphate and shaken for 15min (including untreated control). After inactivation cell cultures were centrifuged (6000xg, 5min) and thoroughly washed with phosphate buffer (pH8). Cells were resuspended in BBM and refrigerated in the dark overnight.

1% BBM agar plates were inoculated with 30µl and spread with sterile plastic spreader. The plates were then sealed with Parafilm and stored in the algal Fitotron ® growth room until colonies appeared. Suitable exposure time for EMS was determined by plotting number of survivors against EMS concentration.

2.2.13 Screening for mutants

Colonies formed on plates after UV or EMS mutagenesis, were microscopically checked using an inverted light microscope (Nikon eclipse TE2000-5) for unusual colour or colony morphology compared to the wild type. Any unusual colonies (such *C. emersonii* UV pale

mutant 'PM' Chapter 4.2) were marked with a pen and added to our stock collection for further testing.

2.2.14 Cell wall characterisation (sonication)

To provide a measurement of cell wall strength for comparing strains (or generated mutants) sonication was used. An MSE Soniprep 150 (23kHZ generator with 2mm wide 'exponential' probe) Sonicator (available at the University of the West of England) was used for experiments. 1ml of algal culture ($1x10^6$ cells ml^{-1}) was sonicated on 'full' (amplitude of 16 microns) in an Eppendorf on ice, with an exponential 2mm probe, under different time limits.

10µl samples were taken from the Eppendorf after sonication and placed in an Improved Neubauer haemocytometer to count intact cells. Readings were finally expressed as a percentage compared to a control. Both direct sampling of the sonicated culture and samples with 500ml 2% SDS added to the Eppendorf were examined.

2.2.15 Selection of cell wall mutants using FACS

Prior to cell sorting *C. emersonii* underwent toxicity testing of calcofluor, to ensure cells could survive staining to enable sorting of viable cells based on calcofluor fluorescence. This was carried out by adding 100µl calcofluor at 0.1% (w/v) to 100µl of *C. emersonii* culture (~$1.5x10^6$ cells ml^{-1}). After incubation for 30min, a 20µl sample was taken and diluted in 980µl. 40µl of this dilution was further diluted into 200µl and a final 30µl aliquot of this mixture was plated on 90mm Petri dishes containing BBM 0.1% (w/v) agar.

For isolation of cell wall mutants of *C. emersonii* a UV mutagenized culture of ~$1.5x10^6$ cells ml^{-1} *C. emersonii* (mutagenesis described in Chapter 2.2.11), was subjected to staining as described above. Cells were then sorted using a BD Influx Cell sorter (with help from Dr. Andrew Herman, University of Bristol) using software Spigot 6.1.9 and FlowJo 7.2.4 (accessed on 10/12/10). Cells were sorted based on 'gating' the following criteria; 'healthy/viable' cell size and autofluorescence (inferred by a control culture), and calcofluor fluorescence (emission wavelength 450-488nm).

2.2.16 Pale mutant characterisation

Wild type (WT) and pale mutant (PM) cultures of *C. emersonii* underwent light and culturing experiments described as follows. Light experiments involved cultivation with a light:dark

cycle of 16:8h, under 'high light' (~200µMol m^{-2} s^{-1} in the SANYO 'environmental test chamber') with the other light experiments 'medium light' (~100µMol m^{-2} s^{-1} uncovered flasks), 'low light' (~50µMol m^{-2} s^{-1} flasks covered with neutral density filter 'hats' (StageElectrics)) and in the dark (~0µMol m^{-2} s^{-1} flasks under a cardboard box) on a Gallenkamp orbital shaker in the algal growth room. At each light condition tested, 4 different types of media were used for cultures of WT and PM, 8 flasks in total for each light condition tested. These were 'BBM' (BBM standard media), 'BBM N- (BBM which after 14 days was switched to BBM N-), 'BBM C+' (BBM with added carbon source) and 'BBM C+N-' (BBM with added carbon source, which after 14 days was switched to nitrate free media with added carbon). Cultures were otherwise cultivated under standard conditions (22-25ºC, 100xg, described in Chapter 2.1.3).

2.2.17 Sampling and filtration of Roman Bath water

Thermal waters from the Roman Bath (ST750647) underwent 'bioprospecting' for novel species of microalgae. Two pools were sampled, the 'Kings Bath' (hottest, 46ºC, KB) which flows into the 'Great Bath' (cooler 39ºC, GB). 1l samples of water were taken from the Great Bath and the Kings Bath and analysed by Severn Trent Services, the pH and temperature were measured on site.

The visible filamentous algae were sampled from scrapings taken from microbial mats submerged beneath the water surface. Approximately 90l of water from the Great Bath was gravity filtered through a custom-made filter-unit at three positions around the Great Bath, the water entry point, exit point and opposite (Figure 2.5).

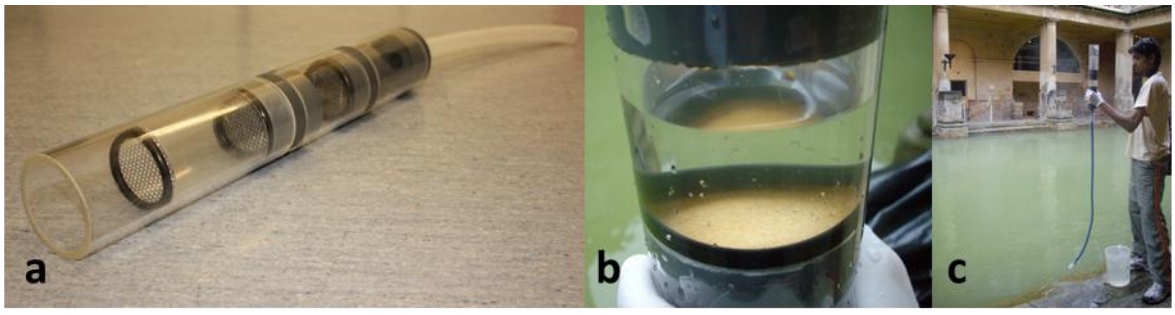


Figure 2.5: The custom made filter-unit for filtration of large volumes of water for isolation of microalgae from the Roman Baths.
(a) the unit comprised of 3 filter sections; coarse (wire mesh grid), medium (washing up scourer) and fine (Whatman GF filter paper). (b) Whatman GF filter paper (finest filter) layer of filter-unit in use at the Baths (orange colouration due to suspended particles in the water from microbial mats). (c) Project student Rishit Shah, filtering Bath water though the filter-unit.

The custom filter-unit comprised of acrylic tube with a coarse 3mm mesh metal filter, a sponge filter and a Whatman GF fine filter paper. The GF filter paper layer was used as a sample for further culturing. When repeated, 300l of water was drawn though an EHEIM compact+ 2000 fish pump from a depth of ~30cm below the surface, in an attempt to capture cells beneath the surface (flow rate of 10l/min for 30min).

2.2.18 Isolation of algae using micropipette

A glass Pasteur pipette was elongated over a blue Bunsen flame and a clean break made at the end (no jagged edges). A well-fitting rubber tube was attached to the other end and a tiny quantity of water sucked into the tube to act as a 'cushion'. Desired cells from scraped samples diluted in dH_2O in a Petri dish (viewed under inverted microscope) were picked up into the elongated pipette using weak suction (tongue) on the end of the tube. All wells of a microwell plate were filled with sterile dH_2O and isolated cells were transferred into one well before being picked up again and deposited into the next well of filtered dH_2O to rinse. The rinsing was repeated again. Final isolated cells were transferred to 7ml BBM:BG-11 medium (1:1) in 15ml screw cap Sterilin vials (adapted from Andersen, 2005).

2.2.19 Isolation of algae using 'filter paper method'

After filtration of water though the custom filter-unit (Chapter 2.2.17) the samples (Whatman GF filter paper discs) were immediately cultured in the laboratory using an adapted technique from Ferris and Hirsch (1991). Sample filter papers were placed on top of a stack of 5 Whatman GF filter papers inside a glass Petri dish with lid (autoclaved) and the 'filter stack' subsequently saturated with 10ml sterile filtered BBM:BG-11 medium 1:1, before sealing with Parafilm. Samples were then left on lab bench (~20ºC and low light ~50µMol m^{-2} s^{-1}) until colonies appeared. Microalgae can grow under a range of light intensities yet for isolation purposes it is best to initially cultivate under low light so as to not further stress the alga. Many species require dark periods for biochemical processes, which if not carried out can be lethal (Dworkin *et al.*, 1992).

2.2.20 Isolation of algae using dilution

Colonies formed on 'filter stacks' were sampled and examined under a microscope. Colonies of interest (green or blue-green in colour) were picked off, diluted and re-plated on filter paper as described in Chapter 2.2.19, in addition to BBM and BG-11 1% (w/v) agar plates.

Where possible (dependent on isolate tolerance to agar), both filter paper and agar methods were repeated until a unialgal culture was achieved. Unialgal cultures were determined by extensive light microscopy.

2.2.21 Isolation of algae using 'plate scoring'

To clean filamentous samples from contaminant fungal species, a method described by Vaara *et al.* (1979) was used. BG-11 1% (w/v) agar plates were scored perpendicular to the end of inoculation with a sterile razorblade. Plates were covered in matt black cloth with opposite end to inoculation site exposed to light source (covered plates placed in SANYO test chamber), to encourage growth of filaments though the agar scores towards the light, shedding fungal and bacterial contaminants (Figure 2.6).

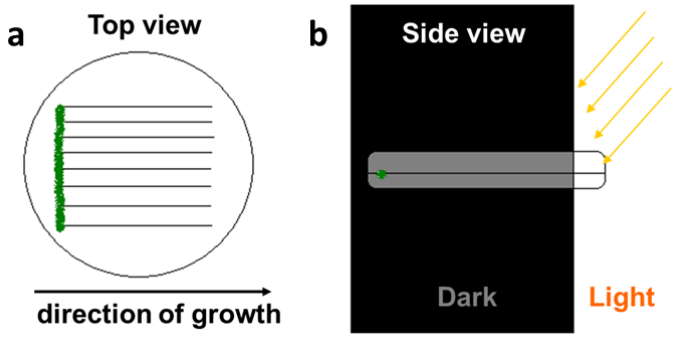


Figure 2.6: Schematic of the 'agar plate scoring' method adapted from Vaara *et al.* (1979).
The top view of the plate (a) shows inoculation to the left (green) and lines representing scores made in the agar by a sterile razorblade. The side view (b) shows the scored plate in darkness with the opposite end (with respect to site of inoculation) exposed to a light source, to encourage the growth of filaments through scores toward the light source.

Clean filaments at the outer boundary ('newest' growth) were sampled after growth had reached the opposite end of the plate and inoculated onto 'filter stacks' (as described in Chapter 2.2.19). This process was repeated until filaments were unialgal and free of fungal contaminants.

2.2.22 Microalgal DNA extraction using 'Beadbeater' and QIAGEN kit

DNA extraction was based on a method by Tiwari (2008). 50ml of algal culture was centrifuged to obtain pellet (5000xg, 10 min in an Eppendorf 5415C centrifuge). Pellet then transferred to an MP Biomedicals lysing Matrix tube, matrices tested: C (red), A (orange), E (purple) and F (white). 600µl of 20mM Na-phosphate buffer (pH8) and 300µl of lysis solution

(10% sodium dodecyl sulphate, 0.5 M Tris HCl (pH8), 0.1M NaCl) was added and disrupted in a bead beater (Savant Fastprep FP120) for 3min (intervals of 45s with 5 min cool down periods) on high speed (6.5). Liquid pipetted into new 2ml Eppendorf and lysate was extracted by adding equal volume of phenol-chloroform-isoamylalcohol (25:24:1), mixed by inverting tubes. Tubes were then centrifuged for 10min at 10,000xg. Upper aqueous layer transferred to clean centrifuge tube (without touching l-l interface), an equal volume of phenol-chloroform-isoamylalcohol (25:24:1) added and centrifuged for 10min at 10,000xg (Ivanikova, 2007). DNA precipitated by adding an equal volume of cold 70% ethanol followed by centrifugation (10,000xg, 5min) and washing with 3 rounds of 70% ethanol, with a final step of pipetting out ethanol and drying under laminar flow hood before resuspension in 50µl of dH_2O.

The QIAGEN plant DNeasy kit (protocol as per kit instructions) was also tested for isolation of algal DNA but proved unsuccessful in isolation of sufficient algal DNA (see Chapter 5.2.4).

2.2.23 Microalgal DNA extraction using a bench drill

The following method was adapted from Tiwari (2008). 50ml of culture was centrifuged (5000xg, 3min, in an Eppendorf 5415C centrifuge) to obtain a pellet and transferred to a 2ml Eppendorf, centrifuged (10,000xg, 1min) and remaining media removed from pellet. 300µl of 20mM sodium phosphate buffer (pH8), 150µl of lysis solution (10% sodium dodecyl sulphate, 0.5 M Tris HCl (pH8), 0.1M NaCl) and enough 0.1mm silica/zirconium beads to make a paste, was added and ground on ice using a bench drill for 5min. 300µl DNA extraction buffer (0.2M Tris-HCl, 0.25M NaCl, 0.025M EDTA) was added and centrifuged 10,000xg for 1min, then supernatant was transferred to a fresh Eppendorf. DNA extraction buffer step then repeated. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to sample, mixed well and centrifuged for 3min at 10,000xg (Ivanikova, 2007). The upper aqueous layer was transferred to clean centrifuge tube (without touching the liquid-liquid interface). Phenol:chloroform:isoamylalcohol extraction was then repeated. DNA was precipitated with an equal volume of cold 70% ethanol and then washed with 3 rounds of 70% ethanol centrifuging (10,000xg, 5min) with a final step of pipetting out ethanol and drying under laminar flow hood before resuspension in 50µl of H_2O.

2.2.24 Polymerase chain reaction (PCR)

Adapted from the Promega ® 'Green Mastermix' product guide. 0.5-1µl of template DNA (unless otherwise stated), 12.5µl Fermentas DreamTaq Green Master Mix, 1µl forward primer, 1µL reverse primer, 5.5µl DNAse free water (algae use 8.5-9µl) were added to a PCR tube, mixed well and spun down using a tube-strip 'Picofuge' (Stratagene). Tubes were then placed in heat blocks of an MJ Research Peltier Thermal Cycler PTC-200. Cycling conditions as follows (unless otherwise stated). 95^{o}C for 5min followed by 32 cycles; 95ºC 45s, * ºC 45s, 72ºC 120s (where * is the melting point inferred by the primer used). After cycling a final extension time of 10min at 72^{o}C took place.

2.2.25 Agarose gel electrophoresis and clean-up

Agarose electrophoresis gel was prepared in 400ml samples and reheated for preparation of new gels. To 400ml 1x TAE buffer, 4g of Invitrogen UltraPure electrophoresis grade agarose was added. This was mixed and microwaved using a domestic 800W microwave (power 7, 10min with occasional swirling) until all particles had dissolved. For every 100ml of gel used 3µl ethidium bromide was added and mixed into gel. Molten gels were poured into moulds and allowed to set at room temperature for 40min.

Solidified gels were then transferred to gel tanks (Bio-Rad) containing enough 1x TAE buffer to coat gels and well combs removed. Samples were then transferred to wells, with the first well kept for a sample of Promega 1kb ladder to determine fragment size. Gel tanks were connected to Bio-Rad Power Pac 300 and ran for 40min at 200mA and 90V. Gels were viewed under a UV transilluminator (Vilber Lourmat TFX 20MC). Bands cut using sterile razorblade and transferred to clean preweighed 2ml Eppendorf.

Clean-up was performed as described by Promega Wizard ® SV Gel and PCR Clean up System kit, as follows (all solutions supplied with aforementioned kit). 10µl membrane binding solution per 10µg of gel slice was added to tube containing sample and incubated in water bath at 50-65ºC until gel slice had completely dissolved. SV minicolumn was placed into collection tube (both supplied with kit) and dissolved gel mixture transferred to this assembly and incubated at room temperature for 1min. Column assembly then centrifuged at 13,000xg for 1min (Eppendorf 5415C centrifuge) to allow DNA to bind to membrane. Flowthough discarded and minicolumn reinserted into collection tube. 700µl of membrane

wash solution (with added ethanol) was added to minicolumn and centrifuged at 13,000xg for 1min, flowthough discarded and minicolumn reinserted into collection tube. This step was then repeated with 500µl of membrane wash solution centrifuging for 5min. The collection tube was emptied and minicolumn reinserted and centrifuged (13,000xg for 1min) with lid off to allow for evaporation of residual ethanol. Minicolumn was transferred to new 2ml Eppendorf tube, 30µl nuclease free H_2O added to minicolumn and incubated at room temperature for 1min. Sample then centrifuged at 13,000xg for 1min. minicolumn was discarded and sample stored at 4ºC until sent for sequencing.

2.2.26 Identification of Roman Bath isolates

DNA sequences checked manually, corrected and assembled using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Sequences were assembled using corrected sequences were used to interrogate the NCBI online database using BLAST. The attribution of genus and/or species identity to the isolates was based on the BLAST total score. Total score is not included in the results (Table 2) as it summarises and compares all data from resultant matches for a single query (and is an arbitrary value for each sequence analysed). Instead 'query coverage' and 'max ident' better describe the 'quality' of each final match (Table 2). In order to be consistent 'total score' from the BLAST outputs was used as a means of identification. Where scores were ambiguous, decisions were based on morphology at the light-microscopy level. This is discussed further in Chapter 5.3.2.

2.2.27 Temperature tolerance experiments

Temperature tolerance experiments were carried out on microalgae isolated from the Roman Baths. Included in these experiments was our 'standard' alga *C. emersonii*. A preliminary experiment was carried out in order to determine the range of temperatures to be tested (space and time restrictions meant a high, medium and low value for temperature had to be determined). This was carried out by inoculating each isolate into 15ml screw cap Sterilin tubes filled with 7.5ml of preferred medium for each isolate (three replicates for each temperature). 'Preferred media' was inferred by DNA 'barcoding' (where possible) and morphology if the isolate had not yet been identified (i.e. BBM for eukaryotes, DM for the diatom and BG-11 for cyanobacteria). Temperatures of 20-60ºC were investigated (in increments of 5ºC) and regulated using heat blocks (Techne Dri-Block DB 3A and 2A) (Figure 2.7). Heat blocks were positioned in the algal growth room, yet due to position in the

heat blocks and the dark screw caps, light inside the tubes would have been low (~50µMol m^{-2} s^{-1}). Each tube was gently inverted twice daily and caps loosened to allow gas exchange. Growth was assessed by comparing samples across temperature ranges. As a result of the preliminary experiment 20°C, 30°C and 40°C were chosen for further experiments.

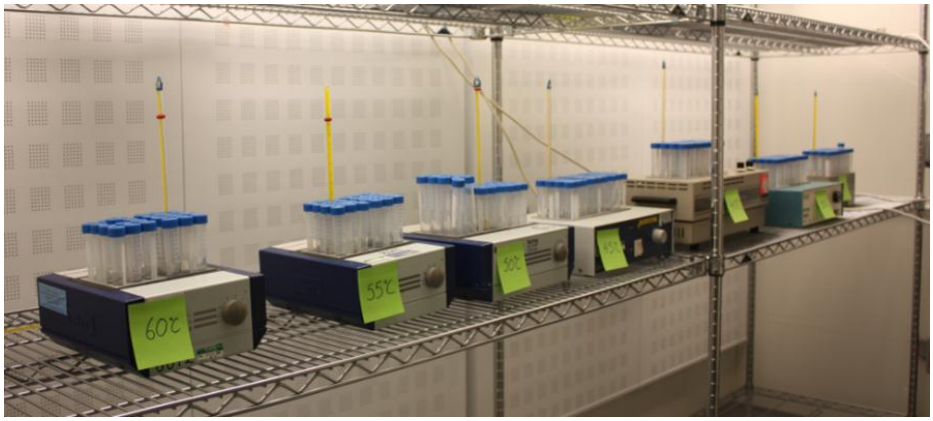


Figure 2.7: The preliminary temperature tolerance experiment carried out to determine an appropriate range of temperatures to further investigate the behaviour of isolated algae.

Each species was cultivated in 2 x 250ml conical flasks with 100ml of suitable medium: BBM for eukaryotes, BG-11 for prokaryotes and DM for *Hantzschia* sp. The flasks were inoculated with 100µl of stock cultures. Cultures were incubated in SANYO test chamber with a lowered light setting of 100-150 µMol m^{-2} s^{-1} on a 12:2h light:dark cycle. *Choococcidiopsis thermalis* and *Hantzschia* sp. culture flasks were covered in a double layer of neutral density filter (StageElectrics, Bristol, UK) due to their sensitivity to light (Figure 2.8). After 12 days one flask of each culture was pelleted and re-suspended in an appropriate nitrogen-free mediaum and cultivated for a further 6 days. Samples were then pelleted by centrifugation (3000xg, 5min in an Eppendorf 5810R centrifuge) and lyophilised.

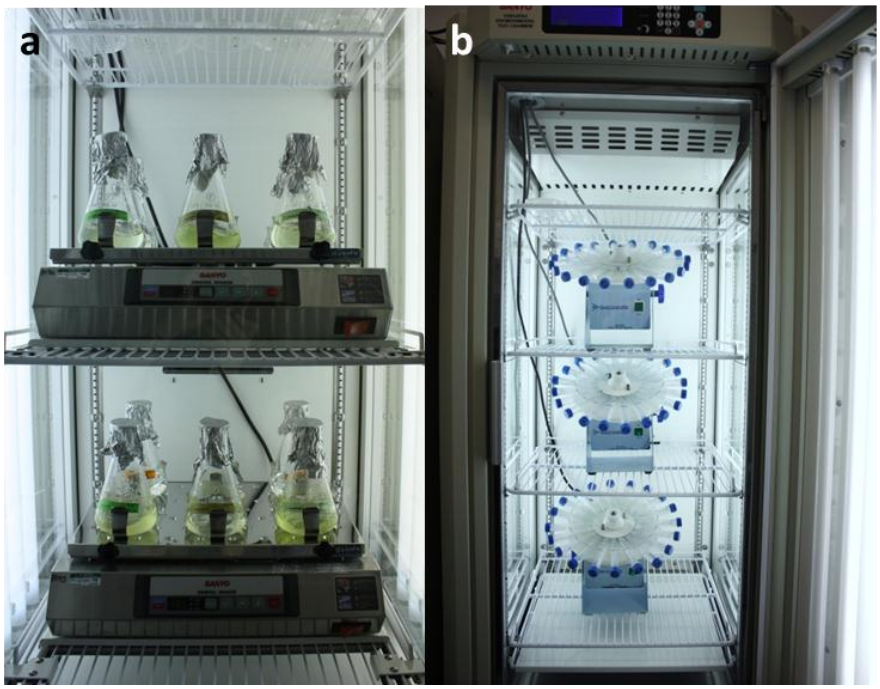


Figure 2.8: Cultivation of algae in the SANYO 'environmental test chamber'.
(a) 'Standard' cultivation of *C. vulgaris* and *C. emersonii* (b) continuous mixing of filamentous microalgal samples to determine an approximate growth rate based on dry weight.

For single celled algae, growth measurements were recorded as cell counts using a haemocytometer and dry weights calculated by lyophilising 2ml of the culture. Filamentous algae had a tendency to form mats or aggregates in the flasks and to stick to the walls of the vessel. To measure growth of filamentous algae it is typical to measure dry weight (Ray and Bagchi, 2002 and Gribovskaya *et al.*, 2007). Consequently, growth measurements for filamentous algae were done by initiating cultures in pre-weighed 15ml falcon tubes containing 7.5ml media. The algae were continuously mixed using a Stuart Scientific SB1 bloodtube rotator (Staffordshire, UK) (Figure 2.8). At 3 day intervals 3 tubes were removed, centrifuged to remove water and lyophilised. FAMEs from these samples were extracted using the 'beadbeating method' outlined in Chapter 2.2.28, and analysed by GC-MS.

2.2.28 Lipid extraction and transesterification methods

All lyophilised algal samples were ground with a pestle and mortar and weighed to the nearest 0.1mg prior to extraction. For the investigation into extraction methods (Chapter 7) 0.1000g were used per sample and 3 replicates were made. Extraction methods used for this experiment are summarised in Table 2.10.

Table 2.10: Summarised protocols for various lipid extraction methods used to extract lipids from *C. emersonii*.

Extraction method	Description
Soxhlet	Algal sample placed in a cellulose tube in soxhlet. Into round bottom flask 100ml $CHCl_3$ and 50ml MeOH (2:1) was added. Extracted at 80°C for 24 h until the solvent leached colourless. Dry solvent from sample. Followed by transesterification.
Beadbeater	1.5ml H_2O added to algae in MP Biomedical matrix E (purple) tubes. Sample beated on setting 6.5, for 3 min in intervals of 45 seconds. Followed by 'Bligh and Dyer' extraction and transesterification.
Sonication	5ml H_2O added to algae in 50ml falcon tube on ice. Sonicated with an amplitude of 15 microns, pulsed 1 minute on, 1 minute off (over 5 min) using an MSE Soniprep 150 with 2mm probe. Followed by 'Bligh and Dyer' extraction and transesterification.
Microwave (1,2)	A domestic 800W microwave was used at 50% power (400W) adapted from (Patil *et al.*, 2010). Samples microwaved over 4 min including a 1 minute interval, in a 200ml Schott bottle with loosened lid, swirling every 30 seconds to mix. Extracted using 2 methods as follows: (1) (modified from Lee *et al.*, 2010) 5ml H_2O added to algal sample, followed by 'Bligh and Dyer' extraction and transesterification. (2) 5ml H_2O added to algae sample with 0.5ml 18M H_2SO_4, followed by 'Bligh and Dyer' extraction.
Anton Paar MAS24 Monowave 300(1,2)	Extractions performed as follows using the Anton Paar MAS24 Monowave 300 reactor: (1) 2ml $CHCl_3$ and 1ml MeOH (2:1) added to algal sample in Anton Paar reaction tube and microwaved for 20 min at 120°C. Followed by transesterification. (2) 2ml $CHCl_3$ and 1ml MeOH (2:1) and 3 drops of 18M H_2SO_4 added to algal sample in Anton Paar reaction tube and microwaved for 20 min at 120°C. Followed by washing and solvent extraction.

N.B: Beadbeater, sonication and microwave extraction (1,2) were all followed by Bligh and Dyer extraction prior to transesterification, with the exception of 'Microwave 2' and 'Anton Paar 2' where no subsequent transesterification was carried out.

'Bligh and Dyer' extraction was performed as follows; for each 10ml of liquid sample 15ml MeOH and 35ml $CHCl_3$ was added and the sample vortexed. Sample then centrifuged at 3000xg, 5 min. Bottom layer removed to round bottom flask. 20ml $CHCl_3$ added to remaining sample and vortexed (to remove remaining oils) and then centrifuged again. Bottom layer removed and added to round bottom flask. Solvent was evaporated. Based on the Bligh and Dyer method (1959).

Transesterification was carried out by adding 20ml MeOH and 1ml 18M H_2SO_4 to dry samples and refluxing for ~4h at 80°C in a heating mantle with stirrers (Electrothermal, Figure 2.9). Wash and solvent extraction was then performed using minimal chloroform and 50ml H_2O. H_2O phase wasremoved and remaining solvent was evaporated again. Final dry transesterified samples were then dissolved in a recorded quantity of dioxane (between 100µl-2ml) and transferred to GC-MS vials.

Figure 2.9: The temperature controlled heating mantle with condensing ring and stirrers used for the transesterification of extracted algal lipids.

For Roman Bath isolate temperature experiments (Chapter 6.2.1) and *C. emersonii* 'Pale Mutant' culturing experiments (Chapter 4.3.2), the 'beadbeating method' was used to extract lipids, as the extraction method experiment had shown it to extract the highest quantity of oil and was quickest, compared to the other methods tested. Composition of resultant biodiesel was measured by GC-MS, as an accurate way to measure FAME profile (Xu *et al.*, 2006).

2.2.29 Gas chromatography mass spectrometry (GC-MS)

FAME extracts dissolved in dioxane were analysed by an Agilent 7890A Gas Chromatograph with capillary column (60m × 0.250mm internal diameter) coated with DB-23 ([50%-cyanpropyl]-methylpolysiloxane stationary phase (0.25m film thickness) and a He mobile phase (flow rate: 1.2ml/min) coupled with an Agilent 5975C inert MSD with Triple Axis Detector. Column was pre-heated to 150°C, temperature held for 5 min and then heated to 250°C (rate of 4°C/min, then held for 2 min). The samples were quantified by comparison to calibrated standards purchased from Sigma Aldrich.

2.2.30 Anton Paar microwave extraction optimisation

Optimisation of microwave extraction was carried out using an Anton Paar MAS24 Monowave 300 microwave reactor. To each G10 (10ml) reaction vial, 6ml of chloroform: methanol (2:1), 0.1ml H_2SO_4 and a magnetic stirrer was added. Samples were ramped (~1°C min^{-1}) to temperatures of 80, 90, 100°C and held at temperature for 2min, 10min, 20min, 2hr.

3. CHARACTERISATION OF THE MICROALGAL CELL WALL

3.1 Introduction

3.1.1 Why study the algal cell wall?

'The critical importance of understanding the cell wall and its relationship to extraction is best exemplified from work on cellulosic feed stocks wherein successful conversion requires advanced knowledge of how cultivation, plant growth and harvesting affects type, structure and amount of cellular carbohydrates' (Cooney *et al.*, 2009).

Most cell wall structures present a significant energy barrier to product extraction from many microalgae (Razon and Tan, 2011). The small cell sizes of microalgae (e.g. *Chlorella vulgaris*, diameter ~5µm) also poses a challenge to extraction, due to a decreased surface area to volume ratio, which increases the energy required when using physical cell disruption methods (Razon and Tan, 2011). For >50yrs *Chlorella* spp. has been seen as a model organism for plant studies due to its simple structure and life cycle, fast growth rates and low nutrient requirements. In the 1970s many experiments were restricted due to the ridged cell wall, with protoplast formation first reported in the 1980s (Urano and Fuji, 2000). As of yet there is no standard method for measuring the mechanical strength of an algal cell wall. However, measuring cell disruption in a French cell press has been reported (Takeda and Hirokawa, 1978; Monsant *et al.*, 2001). The nature of the cell wall can also greatly affect the efficacy of flocculation and other dewatering technologies (Cheng *et al.*, 2007). In addition, the cell wall presents a barrier to to the introduction of DNA into cells, complicating the genetic study of microalgae (Dunahay *et al.*, 1992).

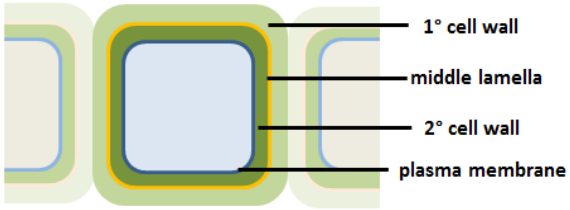
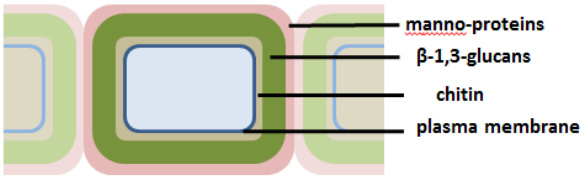
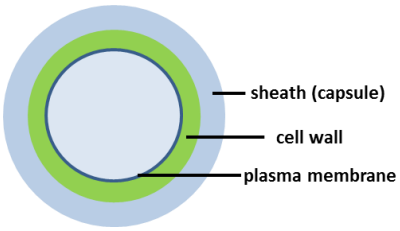
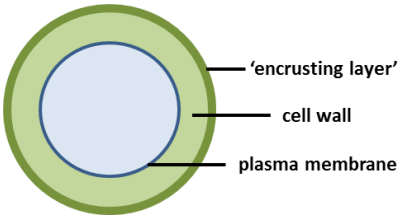
Although enzymatic extractions are unlikely to become viable due to cost they can give valuable clues as to the structure and composition of the cell wall (Parmar *et al.*, 2011). Algae are variable in their biology and physiology (Croft *et al.*, 2006), and are capable of dramatic changes depending on growth conditions (Surek, 2008). As such there are very few descriptions of the cell wall, with those described belonging to a small number of commonly studied species, which are often large celled (i.e. *Haematococcus* spp.) (Hagen *et al.*, 2002).

3.1.2 The algal cell wall

The cell wall is a complex environment. Macromolecular interactions within the cell walls of vascular plants have been extensively studied. However, algal cell walls are markedly more

variable in their composition and morphology (Rodriguez *et al.,* 1999), consisting of a matrix of carbohydrate polymers, proteins and other compounds. The neutral sugar fraction of cell wall is primarily comprised of hemicellulose and glycoprotein, with quantitative differences in rhamnose, fucose, mannose and glucose content, significant enough to act as a taxonomical aid (Burczyk *et al.,* 1995). For example, the cell wall sugar composition of *C. vulgaris* (38% galactose, 22% rhamnose, 14% arabinose, 10% xylose, 10% mannose, 8% glucose) has been determined, yet there is no information relating to macromolecular structure (Takeda, 1993). Algal cell walls notably have significantly higher protein content, which may be structural or metabolically active as part of a synthetic system (Northcote *et al.,* 1958). The cell wall determines shape and integrity as well as having other functions in signalling, defence, cell recognition, expansion and differentiation. Due to certain similarities, microalgae are still believed to make a good plant cell models (Wang *et al.,* 2005) (Table 3.1).

Table 3.1: A generalised representation of cell wall structure and composition for commonly studied groups of algae compared to plants and fungi.
Fungi are included in this comparison as they are capable of synthesising enzymes which digest plant cell walls.

	Structure	Composition
Plant		The primary cell wall comprises of cellulose microfibrils with cross linking glycans and pectin, providing strength and flexibility. The middle lamella consists of polysaccharides such as pectin to adhere primary and secondary cell walls.* A secondary cell wall is present in some plant tissues and contains other often stronger biopolymers (e.g. lignin) which confers strength and waterproofing**
Fungus		True fungi do not contain cellulose in the cell wall but do possess a cell wall with distinct layers of chitin, β-1.3-glucans and mannoproteins.***
Cyanophyta		The gram negative cell wall of cyanobacteria consists of a thin layer of peptidoglycans. Between the cell wall and gelatinous outer sheath, there is a secondary lipid membrane made up of lipoproteins and lipopolysaccharides. ****
Chlorophyta		Eukaryotic algal cells have comparable morphology to land plants. Atop the plasma membrane, is a cell wall consisting of celluloses and glycans, with some algae having an additional 'encrusting layer' containing a significant proportion of silica, calcium carbonate or a biopolymer 'algaenan'. ****

Adapted from *(Alberts *et al.*, 2004), **(Campbell and Reece, 2002), ***(Gow and Gadd, 1995), ****(Barsanti and Gualtieri, 2006).

Microalgae are capable of altering their cell wall composition in response to their environment (Cheng *et al.*, 2007). This is notable in symbiotic algae present inside the fungal walls of the lichens. For example, algal cells of *Pteroderma maculiforme* that were free-living as opposed to lichen-bound, had significantly thicker walls, greater storage reserves and small adaptations to the chloroplast structure (Sanders *et al.*, 2005). The 'phenotypic plasticity' of

microalgal cell walls makes the study of them difficult (Surek, 2008). Their already complex nature can render the tools used for studying plant cell walls ineffective in many cases (Croft *et al.,* 2006). Fluorescent stains and dyes are valuable reagents for the study of microorganisms. Staining with dyes may help distinguish some algal groups, and cell wall sugar composition has in the past been used as a means of classification (Takeda, 1988). Yet many groups (e.g. *Chlorella* spp., *Scenedesmus* spp., *Haematococcus* spp.) are unusual in their response to staining (Markelova *et al.,* 2000). Due to this variation other methods of cell wall profiling at present remain inapplicable to algae. For example, the use of monoclonal antibodies for semi-quantitative mapping (comprehensive microarray polymer profiling) of cell wall glycans, pectins, non-cellulosic polysaccharides and cellulose (Moller *et al.,* 2007) or fluorescent *in situ* hybridisation using taxon specific rRNA targeted probes (Ueno, 2008).

This is a challenge when assessing viability of algal cells with fluorescent stains. Fluorescein diacetate (FDA) is a useful vital stain, yet its behaviour in algae is reversed, when compared to other microorganisms. Viable cells (with a strong cell wall) remained impenetrable to the stain, whereas in dead cultures, >90% of cells were stained with FDA (stains esterases which break down lipids) (Markelova *et al.,* 2000). Propidium iodide (indicator of cell death) is also non-functional when used with algae, as the stain fluoresces red at the same wavelengths at which healthy cells emit red autofluorescence of photosynthetic pigments (data not shown). This also poses a problem to visualisation of non-polar lipids with the stain nile red (red fluorescence). Sick or dying cells show severe degradation of chlorophylls and a reduction in autofluorescence (Pouneva, 1997) was therefore a good indicator of cell death (data not shown).

The poor penetration of stains or unusual staining behaviour in microalgae is a common observation and is usually due to cell wall thickness or morphology. The success of nile red staining in the search for oleaginaceous microalgae varies significantly between different species and often requires the addition of large quantities of DMSO (20-30%) and elevated temperatures (40°C) to aid permeation through thick cell walls (Cooper *et al.,* 2010). The use of ‘modern’ stains such as BODIPY, lower the DMSO requirement and are preferentially used for staining of intracellular lipids (where cell survival is necessary). However, BODIPY also strongly stains glycolipids in the cell wall and as such, may be unsuitable for intracellular lipid quantification methods using staining (Cooper *et al.,* 2010).

Cell wall stains however also exhibit unusual staining in particular species of algae. In addition, culture conditions affect metabolism, cell wall structure and hence staining in many microalgae (Liu *et al.,* 2006; Suzuki *et al.,* 1997; Yamada *et al.,* 1987). For example in *Coelastrum cambricum,* cell wall staining with calcofluor-white (with high affinity for β-1,4 and β-1,3 polysaccharides) varies in intensity depending on cell age. Yet some species do not stain at all, with the exception of broken, dividing or dead cells (e.g. *Scenedesmus acuminatus, Synechocystis* sp. and *Anabaena* sp.) (Markelova *et al.,* 2000). Many other stains recommended for algal use have also been reported to produce unexpected results. As such cell wall studies involving staining are restricted to species with normal stain behaviour.

Cell wall studies have been conducted on algae using enzymes. These studies worked with specific strains and often involved a long treatment (~3hrs) in 'cocktail' of enzymes to produce protoplasts (including cellulose, achromopeptidase, cellulysine, macerozyme, chitosanase, xylanase and pectinase), subsequently ruptured with the addition of a surfactant and presented as a ratio of osmotically labile cells to a control (Honjoh *et al.,* 2003). Naturally occurring enzyme mixtures (such as those from the gut of the mollusc *Helix pomatia*), have also been used for the digestion of a few algae (Northcote *et al.,* 1958).

Various strains of *Chlorella vulgaris* have been studied with enzymes in the past and despite cell wall degradation to 'protoplast' level, were also shown to regenerate their cell walls (Honjoh *et al.,* 2003). The ability for *C. vulgaris* to stain has allowed for observation of cell wall synthesis through the cell cycle, which steadily increases in thickness until autospore release, when the cell wall approaches the same thickness as the mother cell wall (17-21nm) (Yamamoto *et al.,* 2004). An experiment using *Chlamydomonas* sp. (another more widely used model) tested isolated autolysins, which proved effective in digesting the cell wall (Liu *et al.,* 2006). However, these enzymes are synthesized at specific points in the cell cycle, and have strong specificity to strain and growth stage (Jaenicke *et al.,* 1987). This is observed in many other species, particularly when cell wall constituents are layered (i.e. a 'weak' cellulose sensitive inner layer and pectinase sensitive outer layer). Similarly changes can be observed when culture conditions such as pH are changed. Alkalinity has been shown to induce cell aggregation and prevent autospore release, as changes in cell wall metabolism cause the cell wall to stretch instead of rupture (Malis-Arad and McGowan, 1982).

A well-documented example of how microalgae alter their outer morphology is observed in cyanobacteria. Under UV and light stress cells secrete sunscreen-like compounds, mycosporine-like amino acids (MAAs) exuded to the extracellular sheath (Pattanaik *et al.,* 2008). UV tolerance correlates well not only with the presence of MAAs but also with the presence of an acetolysis resistant biopolymer previously believed to be sporopollenin. This sporopollenin-like material remains present in the cells of species which synthesise them throughout the whole cell cycle, unlike MAAs which are radiation-stress induced (Xiong *et al.,* 1997). Internal carotenoids are also produced when algal cells are under stress, to protect from photooxidation by 'mopping up' excess radicals and have been linked to synthesis of a 'strong biopolymer' found in the outer walls of many microalgae. The presence of this strong biopolymer is indicated by the inability of many common stains to permeate the cell wall and in recent years has been termed 'algaenan'.

3.1.3 Algaenan

Small spherical algaenan-containing algae are renowned for their chemical and microbial resistance (Allard *et al.,* 2002) and physical strength (Cooney *et al.,* 2009). Algaenan is not very widespread ecologically and is largely absent in marine microalgae (Kodner *et al.,* 2009). Despite algaenan predominantly existing in the green algae, it cannot be considered as a biomarker (Kodner *et al.,* 2009). Algaenan also constitutes a part of organic sediments in aquatic environments (Rodriguez *et al.,* 1999) and as such, algaenan-containing species accumulate mother walls in the culture medium (Burczyk *et al.,* 1999), (Němcová and Kalina, 2000). Due to its resistance to degradation microalgae have been preserved during fossilization (Derenne *et al.,* 1992). Many algaenan-free species also accumulate cell walls in the medium, which could be due to other complex linkages in the wall (Burczyk *et al.,* 1995). Strains capable of synthesizing algaenan are described as having a 'trilaminar structure' (TLS), containing the biopolymer *in addition* to a cell wall (Burczyk *et al.,* 1999).

Algaenan is a polyhydrocarbon exhibiting many analogies with sporopollenin-like materials (Rodriguez *et al.,* 1999): hydrophobicity, insolubility in polar and non-polar solvents, high resistance to many chemical agents (detergents, alkali and acid hydrolysis, biological agents and high temperatures) (Zych *et al.,* 2009). The chemical resistance makes the study of algaenan and sporopollenin-like materials (such as cutin and suberin) difficult (Burczyk *et al.,* 1999). The study of biopolymeric plant cell walls (despite the dissimilarity to microalgae), may offer some insight into how algaenan is structured or can be broken down. Indeed,

algaenan in *C. emersonii* was suggested to have a strong similarity to the cuticular membrane in higher plants (Allard and Templier, 2001).

Sporopollenins are variable in their composition, consisting largely of mixed polymers of long chain fatty acids (C_{18-30}) and phenolics (Scott and Stead, 1994) which are believed to be precursors, yet the exact structure, synthetic pathways and genetics involved with these types of biopolymers are poorly understood (Dobritsa *et al.,* 2009). Cutin and suberin also provide a tough outer barrier to plant cells and are believed to have a similar structure to algaenan (Kontkanen *et al.,* 2009). Cutin largely comprises C_{16-18} ester linked fatty acids, whereas suberin has a polyaliphatic fatty acid domain and polyphenolic domain (Kontkanen *et al.,* 2009). However, algaenan differs from cutin and suberin as it is less homogenous, with proteins and polysaccharides always associated (Rodriguez *et al.,* 1999). Phenolic residues in the sporopollenin matrix are anchored to the cell wall by covalently bonding with carbohydrates present in the cell wall (Scott and Stead, 1994). Similarly in algae, there is evidence that linkages exist between algaenan and glucose C6 in β-1,4-glucan of the cell wall (based on proportions of monosaccharides rather than structural studies) (Rodriguez *et al.,* 1999). Algaenan is no longer considered to be synthesised from carotenoids (Burczyk, 1987), as carotenoid-negative species are still capable of producing it (*Prototheca wickerhami*) (Burczyk *et al.,* 1999). Yet there remains an as yet undescribed relationship, between algaenan and secondary carotenoids (Monsant *et al.,* 2001).

The most comprehensive depiction of algaenan structure has been described by Allard *et al.* (2002). The outer cell walls of algaenan-containing species mostly comprise linear polyesters containing extremely long chain alcohols and acids (up to C_{80}) and extremely long chain dicarboxylic acids up to $C_{120.}$ It is difficult to infer chemical structures and linkages, due to low yields and chemical degradation on extraction (Allard *et al.,* 2002). There appears to be an absence of ether groups in algaenan (Allard *et al.,* 2002) and evidence of ester and aldehyde linkages (Salmon *et al.,* 2009) (Figure 3.1). Despite the presence of functional groups which should render algaenan open to chemical or enzymatic attack, it is believed that the macromolecular structure provides steric protection (Vandenbroucke and Largeau, 2007). In *Botryococcus* sp. it was found that a decrease in external oleic fatty acid formation led to decreased levels of resistant biopolymer in the outer walls, suggesting a link between biosynthesis of the biopolymer and oleic acid formation (Templier *et al.,* 1993). There is also a strong structural correlation between the different botryococcal lipids and their differential

algaenan structures (Allard and Templier, 2000; Simpson *et al.*, 2003; Vandenbroucke and Largeau, 2007). This is further supported by evidence of the incorporation of oleic acid into algaenan is inhibited by the herbicide 'metazochlor' (Couderchet *et al.*, 1996). It is therefore reasonable to suggest that in other green algae containing the biopolymer, the lipid pathway is linked to algaenan synthesis. It therefore follows that macromolecular algaenan structures are likely to vary between different species of algae. Metabolic defects in algaenan-species led to the formation of unusual sugars and the absence of a TLS (Burczyk *et al.*, 1995). Sugar components in the cell wall increase during growth, yet often algaenan content remains the same until autospore release (Takeda and Hirokawa, 1978).

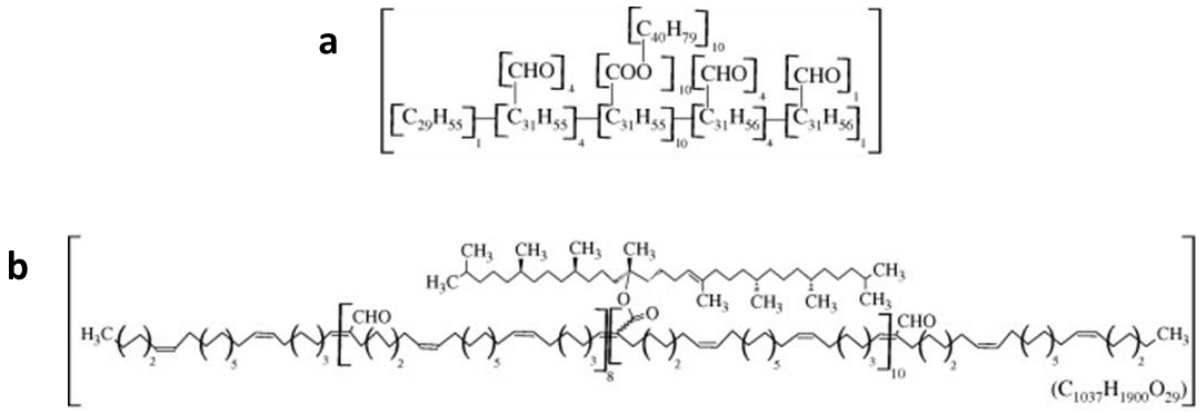


Figure 3.1: (a) Simplified representation of the macro-structure of algaenan and (b) expanded atomistic model of algaenan of *Botryococcus braunii* race L.
There is evidence to suggest algaenan structure varies between different races of *B.braunii* (e.g. race A includes phenolic groups) and as such, algaenan structure is likely to vary between different species of algae (Vandenbroucke and Largeau, 2007). Adapted from Salmon *et al.*(2009).

In plants sporopollenins offer structural reinforcement and help prevent water loss by providing a structure to which waxes adhere, yet foremost it provides physical strength, chemical inertness and resistance to biological attack (Scott and Stead, 1994). The function of algaenan is not clear, although it may simply confer some broad protection and perhaps control permeation of molecules through the wall (Allard *et al.*, 1998). Protection against fungal penetration in symbiotic lichens may also be due to TLS (Derenne *et al.*, 1992). Studies have shown that there is no discernible difference between degradation of algaenan-containing species and algaenan-free species (Afi *et al.*, 1996). Algaenan free strains have been shown to be more sensitive to chemical toxins (Zych *et al.*, 2009).

Algaenan is easily detected by the inability of algal cells walls to stain with common cell wall stains such as crystal violet, calcofluor and sudan IV (Rodriguez and Cerezo, 1996). The low

percentage of stained cells in algaenan-containing species is usually a result of dead or damaged cells (Zych *et al.,* 2009). The presence of a large complex biopolymer such as algaenan can be detectd with FT-IR spectroscopy (Allard *et al.,* 1998) and other chemical techniques such as C-NMR spectroscopy (Burczyk *et al.,* 1995) to determine the structure of fragments (Burczyk, 1985).

Algaenan does not present itself as a simple single layer within the cell wall but as two types of ultrastructure. Algaenan and sporopollenin-like materials can be observed under the transmission electron microscope (TEM), appearing as electron dense and electron translucent layers, also known as 'lamellae' (Derenne *et al.,* 1992) (Figure 3.2). Lamellae in plant cells appear as thicker electron translucent (3-10nm) and opaque layers (10-50nm), yet still bear resemblance to lamellae in algae (electron dense layers 5-6nm thick, electron lucent layers 4-8nm thick) (Scott and Stead, 1994). Algae containing a TLS tend to at a high proportion of their biomass e.g. 13-43% in *Chlorella* spp. Sometimes a TLS can encase a thin cell wall (Derenne *et al.,* 1992). However, a few species with a TLS do not contain algaenan (Allard and Templier, 2000). In *Chlorella fusca,* small plaques of algaenan form on the surface before joining to form a continuous outer coat (Monsant *et al.,* 2001).

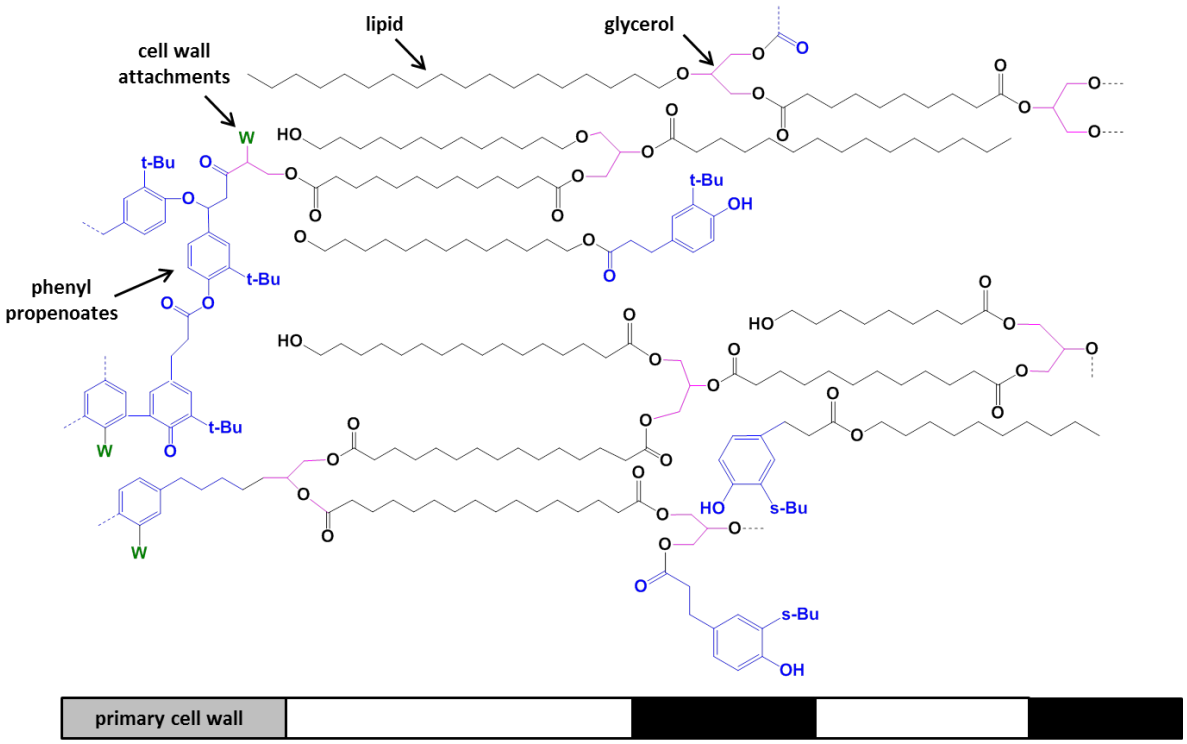


Figure 3.2: Hypothetical structure of suberin (believed have structural homology with algaenan) showing how the chemical structure relates to visualisation of the biopolymer using TEM.
Electron dense (black) and electron translucent (white) regions in suberin are believed to be a result of a layered chemical structure, with alternating phenolic and lipid bands. Adapted from Bernards (2002).

During elongation, ripening and abscission, plants employ families of endo-β-1,4-glucanases to digest materials to modify cell shape or structure (Libertini *et al.*, 2003). Callase (a β-1,3-glucanase), is required for the release of tetrads from pollen grains and may hold the key in degrading sporopollenin (Worral *et al.*, 1992), which could have potential for the digestion of algaenan. Types of acetolysis resistant biopolymeric materials (sporopollenins and algaenans) are not only found in plants and algae but also fungi, the appearance of which predates the first appearance of such biopolymers in plants (Scott and Stead, 1994). Despite the distinct evolutionary separation of fungi from the plant cell lineage and absence of cellulose in 'true' fungi, fungi synthesize enzymes capable of its digestion (Gow and Gadd, 1995), suggesting that although not many existent fungi contain biopolymers such as algaenan they synthesize enzymes for its digestion.

Fungi utilise a lot of 'inaccessible carbon' in the form of woody material, by the secretion of enzymes (Gow and Gadd, 1995) and are also the source of many commercial enzymes used in studies digesting the cell walls of algae. Common fungi (belonging to the basidiomycetes and ascomycetes) have cell walls that contain chitin, β-1,3 and β-1,6-glucans, xylomannoproteins,

galactomannoproteins and α-(1-3)-glucans, and synthesise enzymes capable of their digestion for the fusion of hyphae and regulation of growth forms (Gow and Gadd, 1995).

Cutin can undergo depolymerisation by the cleaving of ester bonds (Kontkanen *et al.,* 2009). Suberin digestion is less well characterised than cutinase depolymerisation, yet both cutinases and suberin-digesting enzymes have been examined from a variety of fungal genera including *Fusarium solani*, *Armillaria mellea* (Zimmermann and Eeemüller, 1984), *Aspergillus nidulans* (Castro-Ochoa *et al.,* 2012) and *Coprinopsis cinerea* (Kontkanen *et al.,* 2009). Cutinase could be used as a biocatalyst for the transesterification of lipids to biodiesel, as its production and activity increases in the presence of olive oils and some TAGs and FAMEs (Castro-Ochoa *et al.*, 2012). This was found for *Aspergillus nidulans* (Castro-Ochoa *et al.*, 2012) and *Fusarium oxysporum* (Pio and Macedo, 2008). Algaenan is believed to have chemical and structural similarities to sporopollenins and suberin (Kontkanen *et al.,* 2009). Fungi from the genus *Fusarium* show low level hydrolytic activity to cutin in addition to lipase activity (García-Lepe *et al.*, 1997), yet some species have shown considerable production of cutin and suberin digesting enzymes, for example *F.oxysporum* (Pio and Macedo, 2008) and *F.solani* (Zimmermann and Eeemüller, 1984). *Fusarium* has also been shown to digest fucoidan (a sulphonated polysaccharide found in algae) (Qianqian *et al.,* 2011), making it an interesting genus to investigate for algal cell wall digestion. The evidence which points towards algaenan being involved with the lipid pathway gives hope that enzymes from fungi could potentially be induced by the presence of algaenan to digest it.

3.2 Results

3.2.1 Growth data

In order to simplify basic culture maintenance and experimentation, growth rates of eukaryotic algal stocks *C. vulgaris*, *C. emersonii*, *S. vacuolatus*, *P. ellipsoidea* ('obi' and 'ni') were determined in BBM and 'Denso media' (recommended medium for *P. ellipsoidea* by Denso) under otherwise 'standard' growth conditions outlined in Chapter 2.1.4. Both cell count (Figure 3.3) and optical density (OD) (Figure 3.4) were measured. OD is an excellent indicator of biomass, yet this requires careful calibration for each species. Cell count is more accurate to monitor growth, as absorbance can change as health or metabolism of the culture changes (i.e. cell flocculation, (data not shown)).

Overall, all strains have faster cell doublings (growth rates) in BBM than 'Denso media', with the exception of *P. ellipsoidea* 'ni' where growth rates in both media were comparable (Figure 3.3). Cell doublings took place approximately every 72h for all strains. The large difference in cell number between *C. vulgaris* and all other strains is due to its significantly smaller cell size (Figure 3.3) ~8times smaller than *C. emersonii* and *S. vacuolatus* (data not shown). Absorbance readings showed that in terms of biomass, *C. vulgaris* accumulated a similar quantity to the other strains (Figure 3.4). Up to day 12 growth rates of all strains under the different media were comparable. After day 12 however, high cell doubling rates continued for strains in BBM, yet were slowed in 'Denso medium' suggesting depletion of a particular nutrient. These results were also reflected in the OD readings (Figure 3.4).

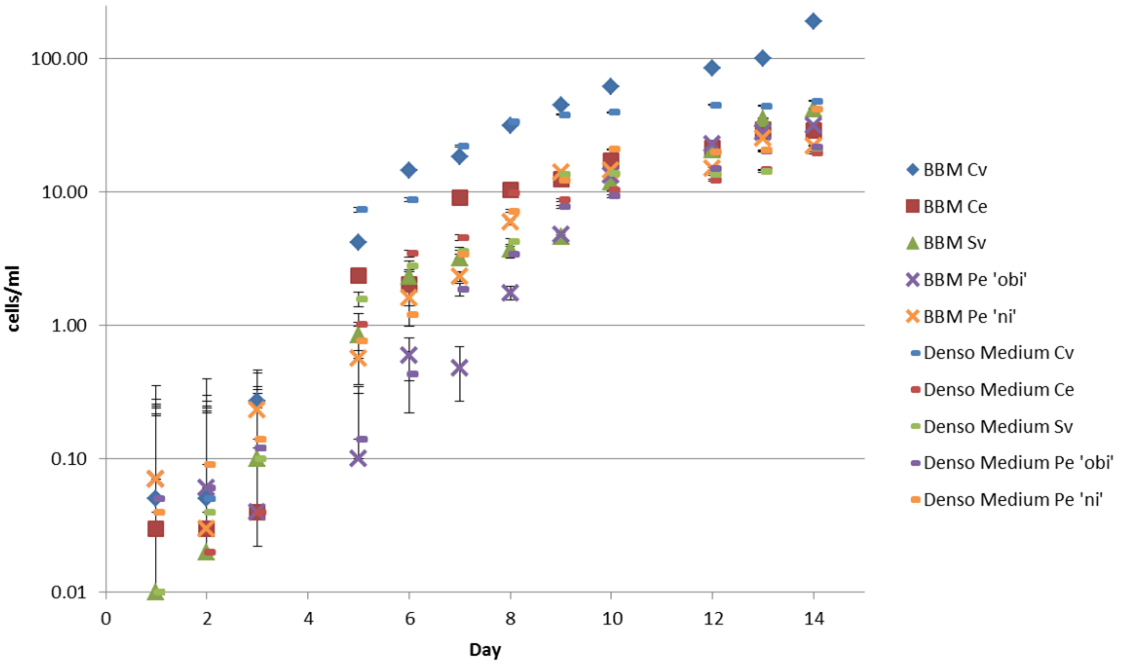


Figure 3.3: Microalgal growth of stock eukaryotic strains (*C. vulgaris, C. emersonii, S. vacuolatus and P. ellipsoidea* 'obi' and 'ni') in both BBM and 'Denso media' under 'standard conditions' measured by cell count using a haemocytometer. Three repeats of measurements were carried out for each measurement, error bars = S.D.

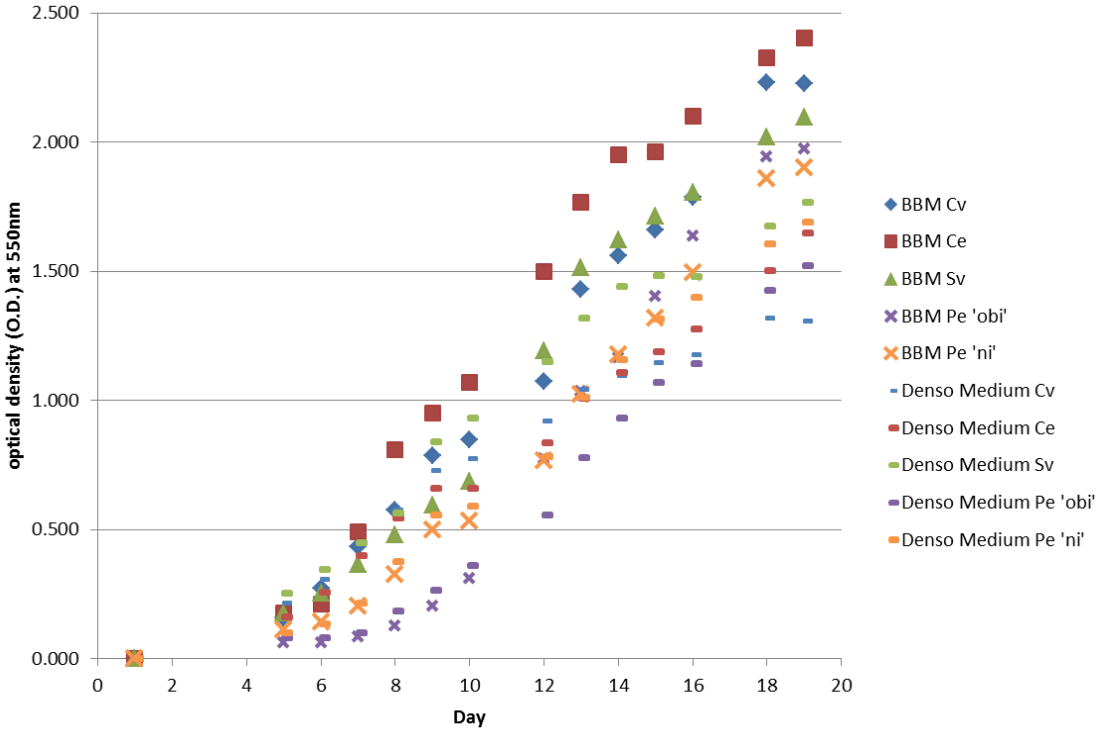


Figure 3.4: Microalgal growth of stock eukaryotic strains (*C. vulgaris, C. emersonii, S. vacuolatus and P. ellipsoidea* 'obi' and 'ni') in both BBM and 'Denso media' under 'standard conditions' measured by optical density(OD) at 550nm using a spectrophotometer. Three repeats of measurements were carried out for each measurement, error bars = S.D.

Growth measurements were repeated in BBM for our 3 stocks *C. vulgaris*, *C. emersonii* and *S. vacuolatus*, measuring growth by cell count, absorbance and dry weight (Figure 3.5). These results were highly comparable to those obtained previously (Figures 3.3 and 3.4). Under standard growth conditions in BBM, *C. vulgaris, C. emersonii* and *S. vacuolatus* had cell doubling times of ~3d (Figure 3.5a) and a comparable pace of 'approximated' biomass accumulation (Figure 3.5b). So as not to greatly reduce the volume in our standard 100ml culture flasks (as this could affect growth characteristics) and to ensure repetition, determination of biomass (Figure 3.5c) was carried out using very small sample sizes. Both *C. vulgaris* and *C. emersonii* were difficult to pellet as cells adhered to the outer walls of Eppendorf tubes and upon removal of supernatant, would be drawn off the walls as a result of liquid surface tension. Due to this and the small sample sizes there was a large error margin, too large to accurately calibrate the absorbance curve to ascertain biomass with accuracy (Figure 3.5b).

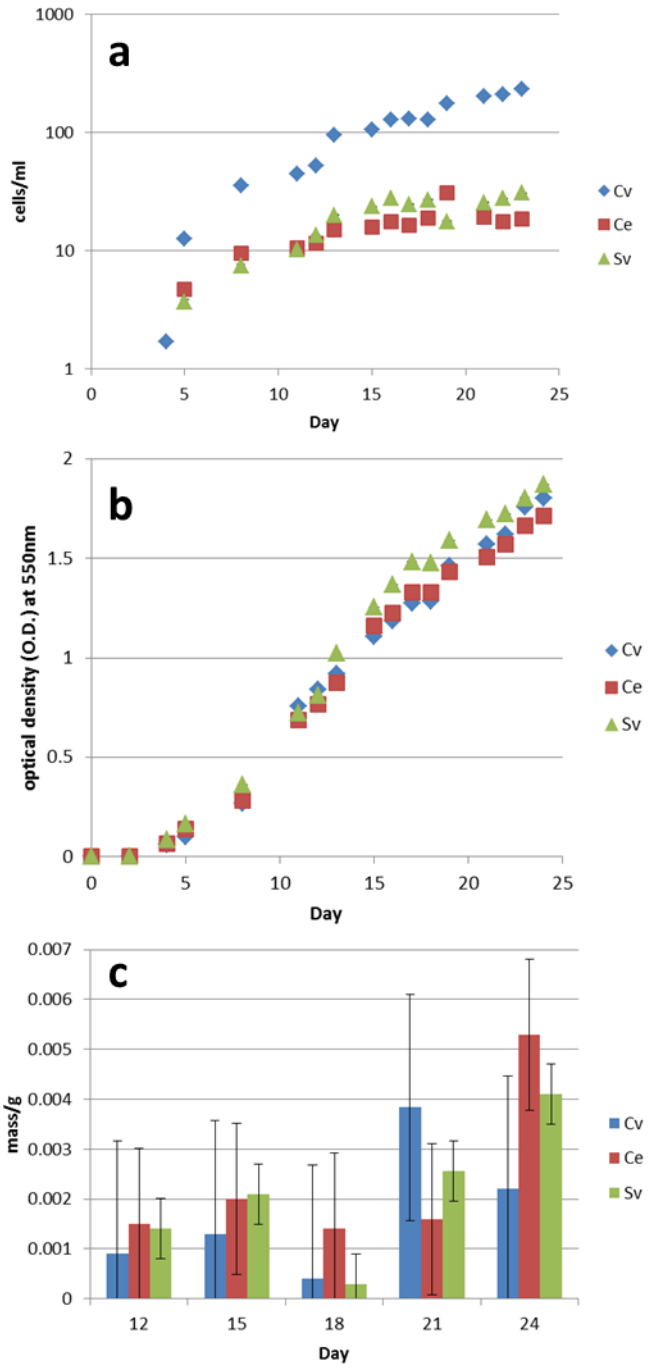


Figure 3.5: *C. vulgaris, C. emersonii* and *S. vacuolatus* cultured in BBM under 'standard conditions'. Growth measured by (a) cell count, (b) absorbance 550nm and (c) dry weight. Three repeats of measurements were carried out for each measurement, error bars = S.D.

3.2.2 Visualising the cell wall using staining

Stains were screened on all strains to determine their behaviour, in particular cell wall stains, with the vision of using staining as a means for detecting changes in the cell wall after enzyme treatment. Firstly the common cell wall stain calcofluor (which stains β1,4 and β1,3-glucans) was investigated on our 5 stock strains (Figure 3.6).

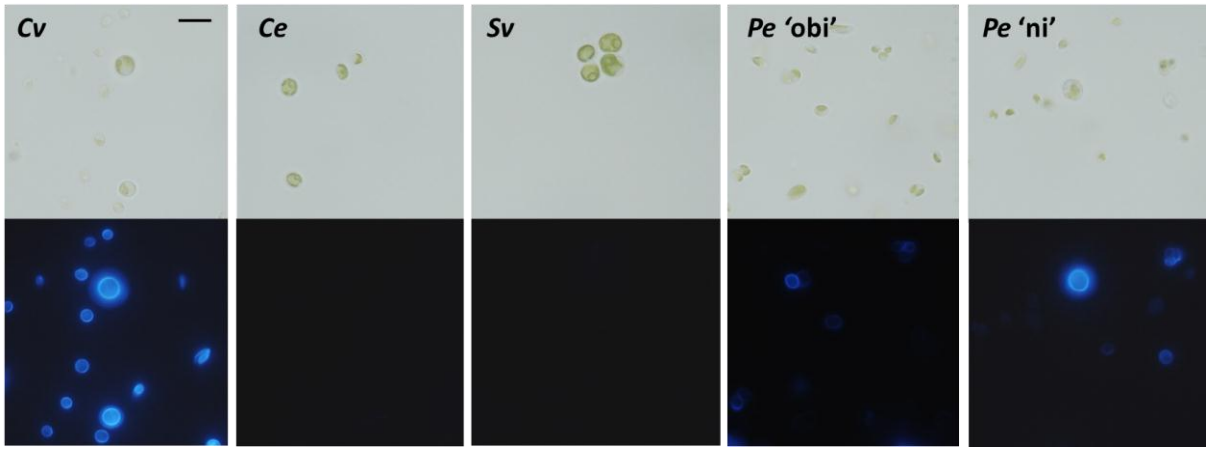


Figure 3.6: Microalgal stock strains (*C. vulgaris, C. emersonii, S. vacuolatus and P. ellipsoidea* 'obi' and 'ni') stained with calcofluor and viewed under the light microscope and confocal (ex488nm).
Cv (*C. vulgaris*) is the only strain to show strong staining with calcofluor. Bar = 20µm.

It is accepted that all Chlorophyta contain some β-polysaccharides in their cell wall yet *C. emersonii*, *S. vacuolatus* did not appear to stain at all and *P. ellipsoidea* 'obi' and 'ni' stained very weakly. These 4 strains did however, show strong staining when the cell wall was broken or damaged, as if calcofluor could leak through an impenetrable outer layer of the wall. The variation in strength of staining was further investigated with *C. vulgaris*, by looking at cell cycle stages to determine if stain intensity changed with stages of the cell cycle (Figure 3.7).

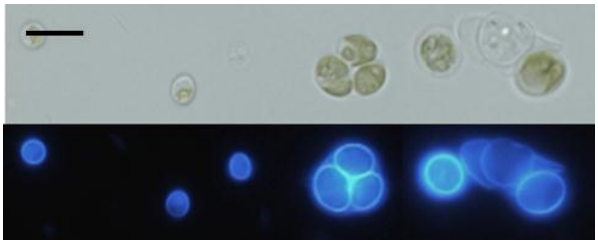


Figure 3.7: Different growth stages of *C. vulgaris* stained with calcofluor under the light microscope and confocal.
Cell size and cycle stage appear to have no discernable differences in staining, with percieved increases in brightness due to layers of cell wall material. Confocal excitation 488nm, bar = 20µm.

Cell walls of *C. vulgaris* showed that there was no change in level of staining during different growth stages. Dividing cells inside the mother cell appear slightly brighter due to layers of stained material (Yamamoto *et al.*, 2004). It appeared that the ability of algae to stain with calcofluor was species specific. As calcofluor could not be used as a means of monitoring cell wall change in algae treated with enzyme mixtures, it was decided to screen other common stains, if only to observe any other unusual staining behaviour. Chapter 2.2.5 outlines the target material or chemical of the commonly used stains tested. Included in the screening of stains were isolated algae from the Roman Baths (discussed in Chapters 5 and 6).

Table 3.2: Summarised stain screening results for all strains.

	Aceto Orcein	**BODIPY**	**Calcofluor**	**Coomassie blue**	**Crystal violet**	**DAPI**	**Nile Red**	**Sudan Black**
Cv	++	++	++	++	++	++	++	++
Ce	++	++*	-	+	-	+*	++	+
Sv	++	++*	-	+	-	+*	++	+
Pe 'obi'	++	++	-	++	-	++	++	+
Pe 'ni'	++	++	-	++	-	++	++	
Hp	++	++*	++	-	-	++*	++	
Os	++	+*	-	+	+	++*	++	-
Mc	++	+*	-	+	-	++	++	-
Ml	++	++*	+	+	++	++*	++	-
K sp.	++	++*	++	++	+	++*	++	+
Cs	++	++*	-	+	-	++*	++	+
Ct	++	+*	-	++	++	++*	++	+
H sp.	++	++	-	++	++	++	++	+

-no staining, + stains target weakly, ++ stains target strongly *also stains the cell wall

Inability to stain with cell wall stains was found for C. emersonii, *S. vacuolatus, C.saipanenensis, P. ellipsoidea* 'obi' and 'ni' and the cyanobacteria *O. sancta, M. chthonoplastes* and *C. thermalis*. As far back as 1985 (Rahat and Reich) it was noted that *C. emersonii* contained a tough biopolymer in its outer wall. The inability to stain cell walls of microalgae is often linked to the presence of this biopolymer (Rodriguez and Cerezo, 1996). This biopolymer is observed in many species and has since been termed algaenan (Burczyk *et al.*, 1999). Algaenan can be visualised using TEM (Chapter 3.2.5).

3.2.3 Enzyme studies

C. vulgaris, C. emersonii, S. vacuolatus responded similarly to enzyme treatments (with the former being most affected and subjected to further testing). All enzymes tested singly and in mixtures (including *Nicotiana* spp. or *Lilium* spp. bud extracts) had little effect on the cell

wall when observed with calcofluor staining and with subsequent addition of SDS to rupture sensitive cells. The discovery of 'algaenan' and its effect on staining and enzymatic resistance its resistance to enzymes, led to one final set of experiments using enzymes on *C. vulgaris* (as an algaenan negative species), *P. ellipsoidea* 'obi' and 'ni' using sensitivity to SDS and a haemocytometer as a means to measure changes or weakening of the cell wall.

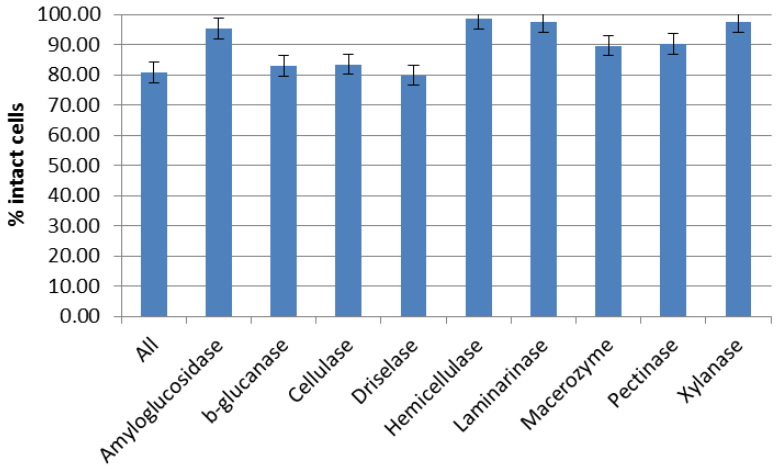


Figure 3.8: *C. vulgaris* cell sensitivity to lysis after exposure to various enzymes (12%w/v, over 3h).
Enzymes were tested singly and as a mixture ('All'). Samples were analysed using a hemocytometer and results expressed as a percentage of intact cells compared to the control. Three repeats were performed for each measurement, error bars = S.D.

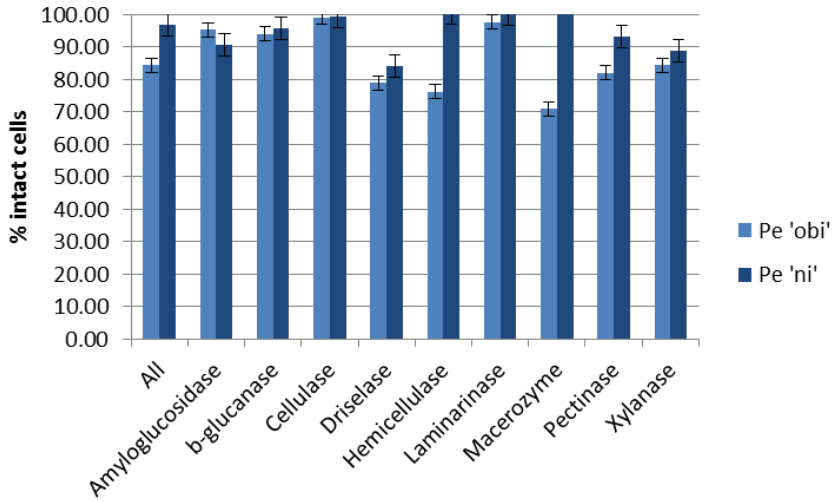


Figure 3.9: *P. ellipsoidea* 'obi' and 'ni' cell sensitivity to lysis after exposure to various enzymes (12%w/v, over 3h).
Enzymes were tested singly and as a mixture ('All'). Samples were analysed using a hemocytometer and expressed as a percentage of intact cells compared to the control. Three repeats were performed for each measurement, error bars = S.D.

C. vulgaris was most sensitive to single enzymes β-glucanase, cellulase, pectinase and natural mixtures 'Driselase' and 'Macerozyme' (Figure 3.8). Affected cells however did not represent

more than 20% of the population, which is perhaps likely due to the affected cells representing a proportion, either undergoing cell division or cell death. The most effective were enzyme mixtures Driselase and 'All' (all listed enzymes). This suggests that despite the lack of algaenan *C. vulgaris* cell walls remain strong and resistant to enzymatic attack. The cell wall of *P. ellipsoidea* 'obi' was marginally more weakened by enzyme exposure than *P. ellipsoidea* 'ni'. Both show similar behaviour when exposed to enzymes, with the exception of Macerozyme which strongly affected 'obi' but not 'ni' (Figure 3.9). Driselase appeared most effective for 'ni'. However, like *C. vulgaris* only a small fraction of cells was ever affected.

3.2.4 Scanning electron microscopy of C. vulgaris and S. vacuolatus

SEM was used to try and identify why in a concentrated culture, cells of *S. vacuolatus* began to autoflocculate (unlike our other strains). Autoflocculation is a desirable trait, as dewatering of algal cultures adds a large cost to its production. It was found that *S. vacuolatus* cells were encased in a loose 'membrane', creating furrows which may have helped cells stick together (Figure 3.10). In contrast *C. vulgaris* cells were smooth. This could be caused by a difference in osmotic requirements between the species, despite both having been cultured under identical conditions. In addition pH can also affect flocculation of algal cultures due to surface charges (Kaloudis, 2012)

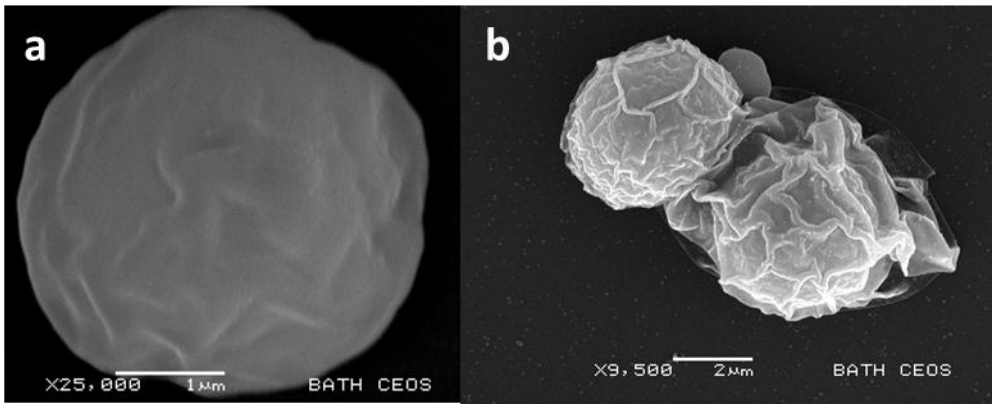


Figure 3.10: SEM images of (a) *C. vulgaris* and (b) *S. vacuolatus*.
(a) cell of *C. vulgaris* showing a smooth outer cell surface. (b) cells of *S. vacuolatus* showing a wrinkly cell surface. Cells of *S. vacuolatus* showed loose outer membranous material.

3.2.5 Transmission electron microscopy for visualisation of the cell wall

TEM sections were made of all stock species and Roman Bath isolates (Chapters 5 and 6) to better visualise the cell wall due to the odd staining behaviour exhibited. For *C. vulgaris* and *P. ellipsoidea* samples were prepared and viewed at the University of Bath, with voltage settings on the TEM at 120kV, for good contrast of biological samples with minimal sample damage. *C. vulgaris* had a thicker cell wall than *P. ellipsoidea* and there was no presence of a TLS (Figure 3.11). There was a visible TLS in *P. ellipsoidea*, which appeared to be the only visible constituent of the cell wall, making it remarkably thin for the overall cell size (Potter, 2009). The TLS is likely to be a biopolymer such as algaenan. In *P. ellipsoidea* the algaenan cell wall not only accumulated in the medium but strongly associated with cells, sticking dividing cells together forming clumps.

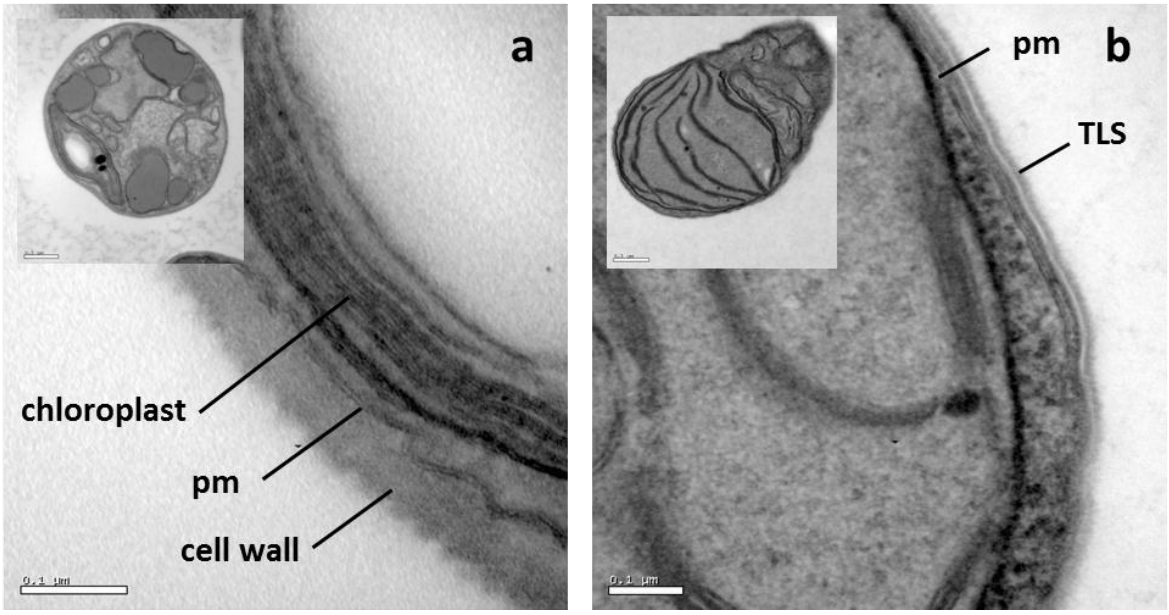


Figure 3.11: TEM images comparing the cell walls of *C. vulgaris* and *P. ellipsoidea*.
(a) *C. vulgaris* with a thick cell wall lacking a TLS and (b) *P. ellipsoidea* with a very thin cell wall comprised exclusively of a TLS likely algaenan. pm = plasma membrane, TLS = trilaminar structure, bar = 0.1µm.

Image quality was not always good for the Roman Bath isolates as moisture was present in the resin curing oven, which had rendered them brittle and difficult to section. In order to save on project budget, Roman Bath isolates were sectioned and viewed under TEM at the Johnson Matthey site in Sonning Common. The settings of their TEM were at a much higher voltage due to the nature of their chemical samples (voltage 200kV). As protection against the higher voltage used for viewing samples were carbon coated prior to viewing. The carbon coating and higher voltage resulted in poorer contrast and added granularity to the images. Despite

carbon coating sections also began to show 'bleaching' after prolonged periods of viewing, 'burning' samples under the electron beam. This accounted for differences in image quality shown in the Figures.

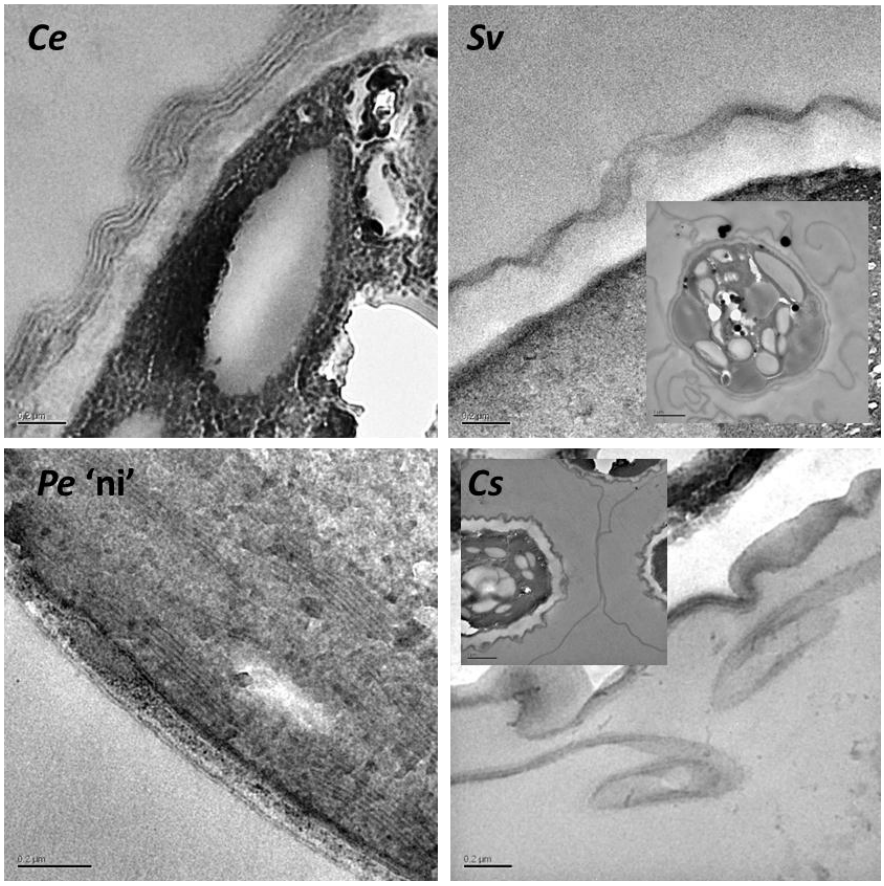


Figure 3.12: TEM images of *C. emersonii, S. vacuolatus, P. ellipsoidea* 'ni' and *C. saipanensis*.
All these are green eukaryotic algae which exhibit a TLS structure in the outer wall, likely algaenan. Inserts are to show the accumulation of algaenan-containing cell walls in the media which are strongly associated with cells. Bar = 0.2µm.

TEM images of green eukaryotic algae which showed poor staining with cell wall stains such as crystal violet and calcofluor, confirmed the presence of a TLS (Figure 3.12). This suggested the presence of the biopolymer algaenan. Inserts show accumulation of old cell walls, which remained closely associated with cells. All the algaenan containing species showed differences in the cell wall structure. *C. emersonii* showed multiple layers of electron dense and electron translucent layers (TLS) closely packed at the surface of the cell in addition to a thick cell wall underneath. *S. vacuolatus* had a single layer of what appeared to be an amorphous 'unraveling' TLS on top of a thick cell wall; this is also observed with *C. saipanensis. P. ellipsoidea* only possessed a single thin layer of TLS and apparent absence of a cell wall.

TEM images of the remaining Roman Bath strains revealed diverse cell wall morphologies (Figure 3.13). Species which did stain such as *Klebsormidium* sp., *M. chthonoplastes* and *M. laminosus* appeared to have thin walls with no apparent biopolymer. Those which showed unusual or poor staining *O. sancta, M. chthonoplastes* and *C. thermalis* all had thicker walls comprising of two or more layers and or a mucilaginous sheath. In some of these layers, fibres could be seen.

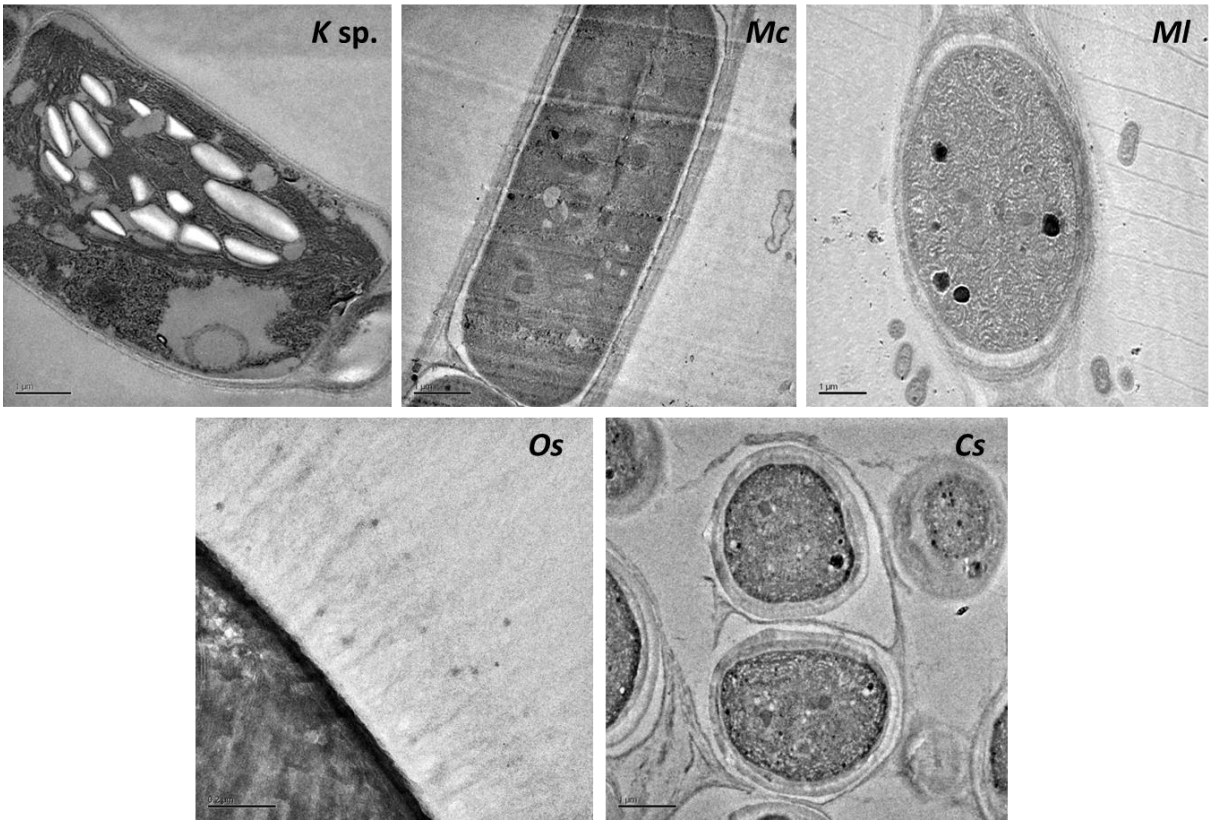


Figure 3.13: TEM images of *K. sp., M. chthonoplastes , M. laminosus, O. sancta* and *C. thermalis*.
These species do not exhibit a TLS structure in the outer wall. Some cells have thick walls comprising two or more layers. Bar = 1µm (with the exception of *Os* where bar = 0.2 µm).

3.2.6 Measuring cell wall strength

Ultra-sonication relies on the high frequency generation of vibrations which generate the formation of bubble or 'cavities' which violently collapse, which when in proximity to cells causes physical damage (Santos *et al.*, 2009). Cell wall strength was measured using a sonicator, comparing strains sonicated over different time periods both with and without subsequent addition of SDS to lyse damaged cells. Measurements were given as percentages of lysed cells compared to a control (Figure 3.14).

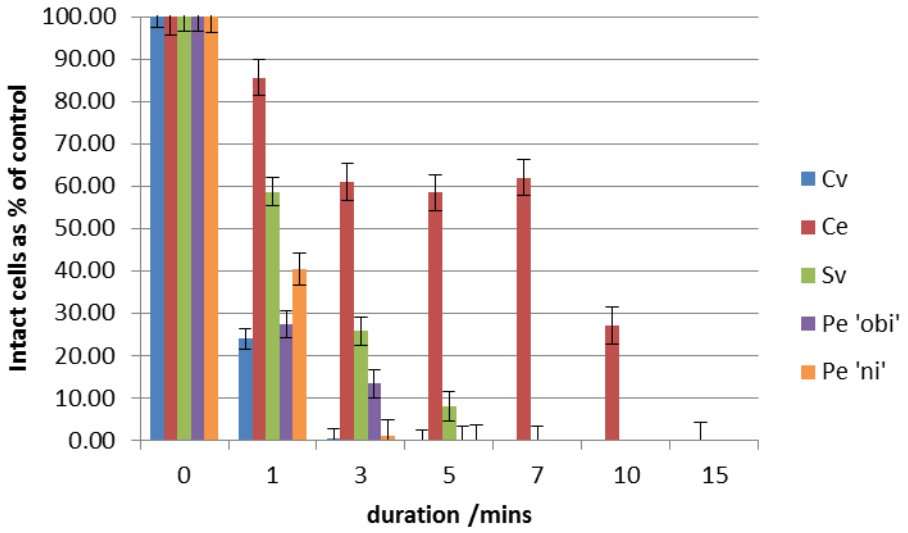


Figure 3.14: Effect of ultrasonication on cell wall integrity for *C. vulgaris, C. emersonii, S. vacuolatus, P. ellipsoidea* 'obi' and 'ni'.
Samples were viewed using a hemocytometer and values are expressed as percentage of intact cells compared to the control. Three repeats were performed for each measurement. Error bars = S.D.

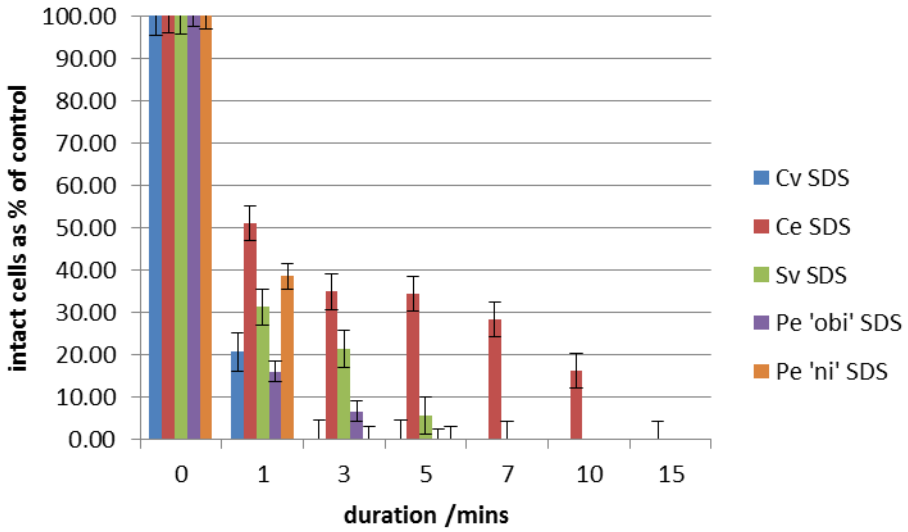


Figure 3.15: Effect of ultrasonication on cell wall integrity for *C. vulgaris, C. emersonii, S. vacuolatus, P. ellipsoidea* 'obi' and 'ni' and the subsequent addition of SDS to lyse intact cells with cell wall damage.
Samples were viewed using a hemocytometer and values are expressed as percentage of intact cells compared to the control. Three repeats were performed for each measurement, error bars = S.D.

Using this experiment as an indicator of cell strength, *C. vulgaris* was found to be the weakest followed by *P. ellipsoidea* 'ni' and 'obi' and *S. vacuolatus* (Figure 3.14). *C. emersonii* appeared to have a very strong cell wall taking 15min of sonication at 23 kHz on full amplitude (16 microns) for all cells to become broken. SDS was used to aided the lysis of intact cells which had sustained cell wall damage (Figure 3.15). This is most notable for *C. emersonii* where values of intact cells dropped by approximately a third. Despite the

cooling of reaction vessels with ice, samples tended to feel warm to the touch when sonicated for longer than 3min.

3.2.7 Digestion with extracellular enzymes from Fusarium oxysporum f.sp. elaeidis

Due to the proposed similarity in structure of algaenan to plant biopolymers, plant pathogenic fungi may provide relevant enzymes for further study. In order to assess the digestion of algaenan, it required isolation from other cellular material. Extraction of algaenan was performed on *C. emersonii* and *P. ellipsoidea* 'ni' and essentially involved four steps; alkaline extraction, lipid extraction, saponification followed by acid digestion. Algaenan is then left behind as a residue after all other matter has been degraded and removed with solvents. Residue extracted from *C. emersonii* was brown in colour and black from *P. ellipsoidea* 'ni'.

Peaks characteristic of algaenan were presented in a paper by Allard *et al.* (1998) and showed peaks at 3000, a double peak at 1000-1700 and a peak at 750 cm^{-1}. Algaenan extracted from *C. emersonii* and *P. ellipsoidea* showed a slight shift of the double peak to 1750-2500 cm^{-1}, but otherwise match the FT-IR trace presented by Allard *et al.* (1998) (Figure 3.16 and 3.17). These findings suggested that there is an algaenan-type substance in the extracts from *C. emersonii* and *P. ellipsoidea* 'ni'.

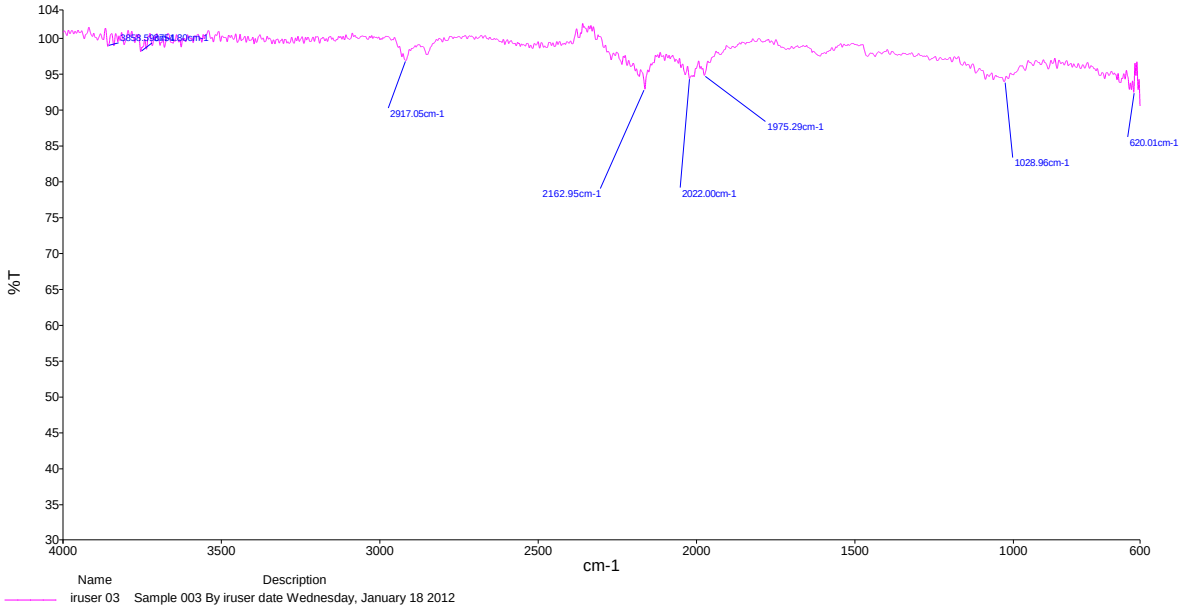


Figure 3.16: FT-IR trace of algaenan extracted from *Chlorella emersonii.*
Peak assignment: 3000-2800cm^{-1} CH_3 and CH_2 stretch vibration, 2500-2000cm^{-1} CH_3 and CH_2 asymmetric bending, 620cm^{-1} long skeletal chain vibrations of $(CH_2)_n$.

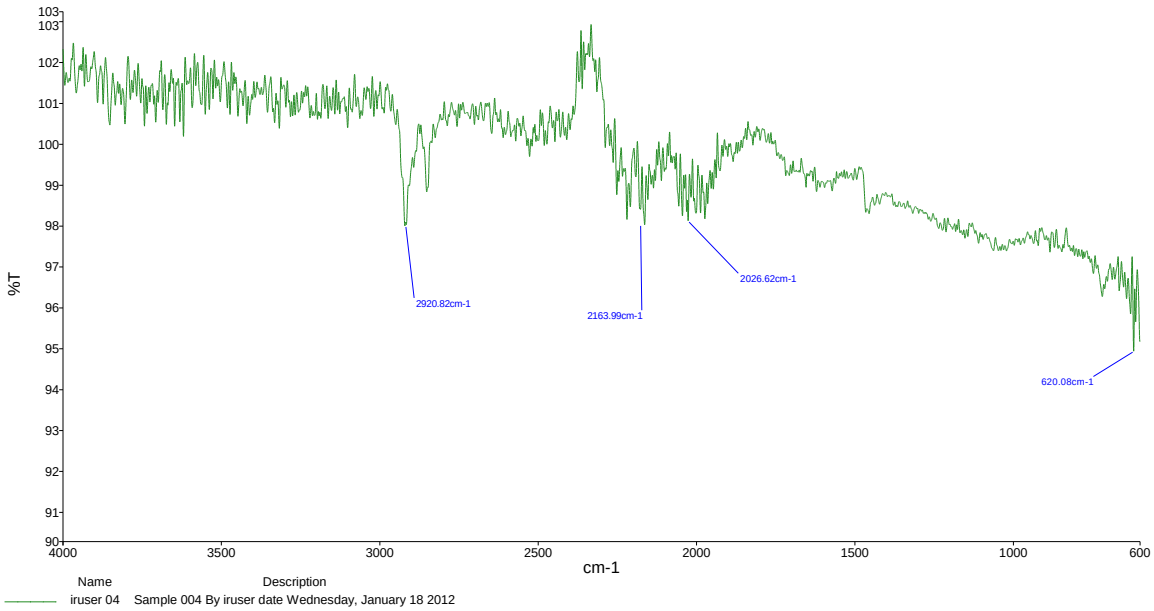


Figure 3.17: FT-IR trace of algaenan extracted from *P. ellipsoidea* 'ni'.
Peak assignment: 3000-2800cm^{-1} CH_3 and CH_2 stretch vibration, 2500-2000cm^{-1} CH_3 and CH_2 asymmetric bending, 620cm^{-1} long skeletal chain vibrations of $(CH_2)_n$.

Due to the availability of *Fusarium oxysporum* and experience in its cultivation at the University of Bath, this species was utilised in the preliminary experiments described below. Despite extraction from ~20g of dry algal material, large sample losses resulted from repeated filtering during algaenan extraction, which resulted in a final quantity of algaenan <5mg. The experimental design was affected by the limited quantity of algaenan. This is reflected in the small volumes used and lack of repeat experiments for algaenan containing samples.

There is no direct assay for the digestion of algaenan. Agar plate experiments were designed in order to observe any changes in 'community behaviour' when a fungus encounters a potential carbon/nutrient source (perhaps algaenan or algae) in a low energy environment. Environmental cues shift the balance between different energy-capturing modes in fungi. During periods of starvation higher incidence of branching may be seen whilst keeping energy expenditure low. Once a food source is encountered, growth and energy reserves are devout toward the direction of the nutrient source (thickening mycelial cords) and regression of non-connective cords, this allows for efficient foraging. This can be observed in many species of fungi (Rayner *et al.*, 1994).

Where *F.oxysporum* sp. *elaeidis* was cultured on agar plates, plates were prepared by adding 1% (w/v) of agar to the media. Three types of media were used with varying quantities of

carbon source (sucrose): C+ (30.0g l^{-1}), Cmin (0.5g l^{-1}) and C- (0.0g l^{-1}). Plates were inoculated centrally with *F.oxysporum* f.sp. *elaeidis* using 10µl of culture and allowed to dry for 5 min in a laminar flowhood. For the investigation into the digestion of the algal cell wall, 10µl algal samples (autoclaved, frozen and live) were dropped approximately 1cm from the edge of the plates. In the case of algaenan, small visible samples (<1mg) were transferred 1cm from the edge of the plate. Plates were incubated at 28°C for 5 days and photographed under the inverted microscope.

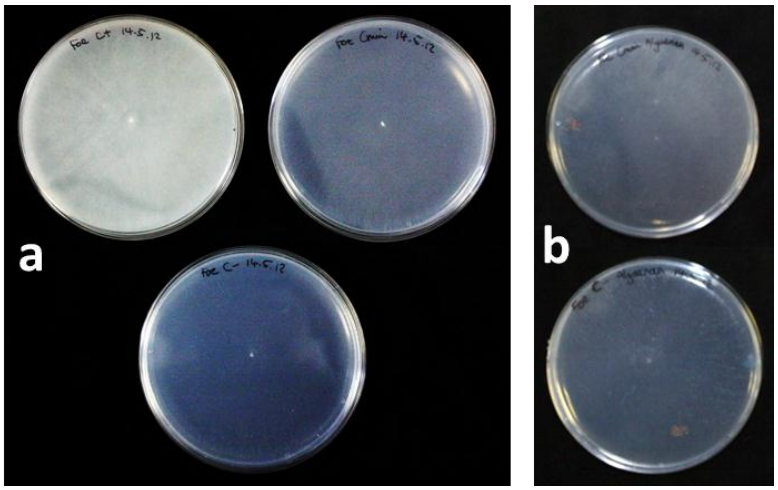


Figure 3.18: *F.oxysporum* f.sp. *elaeidis* cultured on agar plates of differing media.
(a) control agar plates C+, Cmin and C-, (b) cultured on Cmin (above) and C- (below) plates with a sample of algaenan spotted near the plate edge.

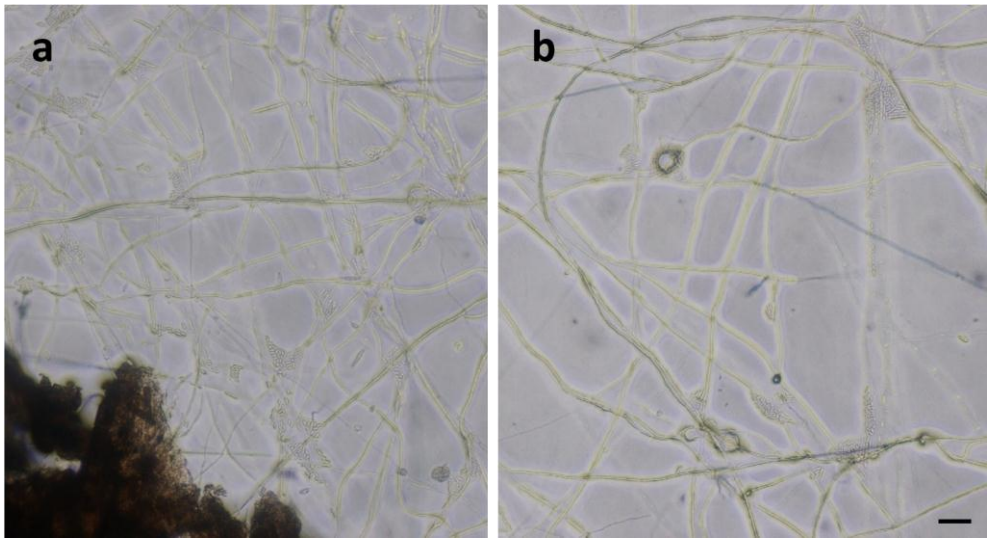


Figure 3.19: Inverted microscope images of *F.oxysporum* f.sp. *elaeidis* on Cmin media.
(a) in the vicinity of the algaenan sample and (b) at another opposite location on the same plate. Bar = 20µm.

All agar plate experiments revealed no changes in community behaviour of *F.oxysporum* f.sp. *elaeidis* on encountering algal material (autoclaved, live or frozen) or algaenan, with plates resembling the controls (Figure 3.18a and 3.18b). There were no observable physiological differences in thickness, shape, colour, density or budding of hyphae using the inverted microscope (Figure 3.19). Density of hyphal filaments increased as carbon content of the agar media increased from C- to C+ (Figure 3.18a).

15ml liquid fungal cultures containing algae (autoclaved, frozen and live) in various strengths of carbon supplemented media were also investigated. Samples were subjected to cell counts (in comparison to controls, should the fungus lyse cells due to cell wall degradation) and were also stained with calcofluor in an attempt to observe any changes to the algal cell wall in response to exposure to *F.oxysporum* f.sp. *elaeidis* (for full methods see Chapter 2.2.10).

As carbon content increased in control liquid cultures of *F.oxysporum* f.sp. *elaeidis* growth rate increased as did final spore count after 120h (Figure 3.20a). *F.oxysporum* f.sp. *elaeidis* cultivated with algae (autoclaved, live and frozen) achieved higher growth rates and cell densities than the controls in both Cmin and C- media (Figure 3.20b and Figure 3.20c). This is likely due to the presence of additional nutrients released from algae that had been autoclaved or frozen. As for the samples containing live algae, algal cells appeared unaffected by the presence of the fungus, yet slightly elevated growth rates of the fungus was observed. This was potentially due to the high proportion of extracellular organic matter in algal cultures, which may have remained present despite prior washing.

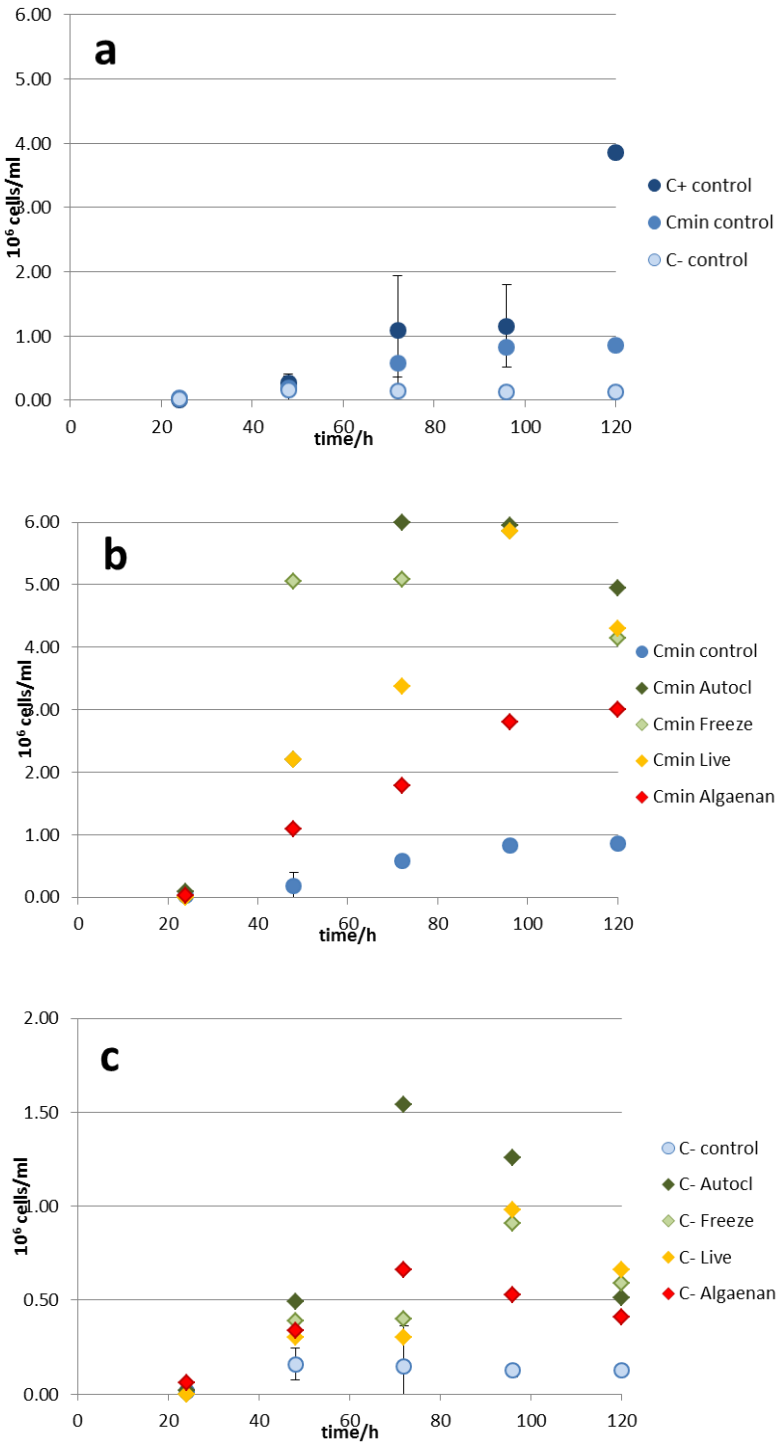


Figure 3.20: Cell counts for growth of liquid cultures of *F.oxysporum* f.sp. *elaeidis*.
Cells were counted using a hemocytometer (a) control cultures in differing carbon media (b) cultures using Cmin media (c) cultures using C- media. Three repeats were performed for each condtion of the controls, error bars = S.D.

F.oxysporum f.sp. *elaeidis* cultured with autoclaved algae had significantly faster growth rates and achieved higher cell densities in both Cmin and C- media (Figure 3.20b and Figure 3.20c). Samples containing visible quantites (<0.1mg/15ml) of algaenan exhibited increased

growth rates and cell densities in both Cmin and C- media samples. However growth rates and cell densities in algaenan supplemented cultures remained lower than those containing algae in Cmin media (Figure 3.20b), and approximately equivalent to samples containing algae in C- media (Figure 3.20c). The visible quantities of algaenan added to samples also did not appear to change over the course of the experiments. Small quantities of each liquid sample were also stained with calcofluor and observed under the light microscope in the hope of detecting changes in staining ability of the algal cell wall. However no changes were detected. Fungal material and autoclaved and frozen algal material stained strongly, whereas live algal cells did not stain.

The principle behind using GC-MS analysis was to detect any degradation products (potentially long chain fatty acids, esters or alcohols) should *F.oxysporum* f.sp. *elaeidis* be capable of digesting algaenan. The supernatant of *F.oxysporum* f.sp. *elaeidis* (cultivated using Cmin) was filtered and tested for its ability to digest algaenan over 24h. This was carried out as follows: *F.oxysporum* f.sp. *elaeidis* cultured in 'Cmin' media was transferred to 15ml greiner falcon tube and pelleted (13,000xg, 5min). The supernatant was transferred to a new 15ml greiner falcon tube and a visible quantity of *C. emersonii* algaenan added (<1mg). The sample was then incubated at 28°C, 100xg. After 24hr the sample was filtered and supernatant retained for further processing and GC-MS analysis. Similarly a control sample of Cmin media (containing algaenan but no fungus) and a sample of *F.oxysporum* f.sp. *elaeidis* (cultivated in the presence of algaenan) was also filtered, transesterified and subjected to GC-MS analysis for comparison. These samples were prepared for GC-MS analysis as follows: to ~15ml filtrate samples 20ml $CHCl_3$:MeOH (2:1) and 1 drop concentrated H_2SO_4 were added into a 50ml round bottomed flask and shaken well. Samples were then transesterified at 80°C and stirred at ~300xg for 3h. Samples were then washed with dH_2O, dried and dissolved in 200µl of dioxane and analysed by GC-MS (full method given in Chapter 2.2.10).

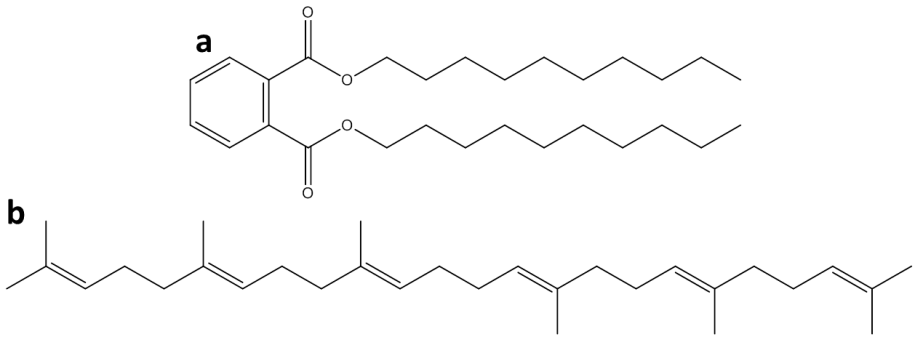


Figure 3.21: Chemical structure of (a) dedodecyl phthalate and (b) squalene detected in GC-MS samples of culture extracts from algaenan digestion experiments.

Long chain fatty acids, esters or alcohols were not detected in any of the samples analysed by GC-MS (data not presented). However two chemical signals identified as dedodecyl phthalate and squalene were detected in the GC-MS for each of the prepared samples examining algaenan digestion (Figure 3.21). As these chemicals were not calibrated for the GC-MS, exact quantities could not be given however, a relative quantitative estimate was inferred by peak area. The control showed only a very small quantity of squalene, which increased ~3 fold in the samples where *F.oxysporum* was cultured in the presence of algaenan and ~4 fold in the sample with algaenan exposed to fungal culture supernatant for 24h.

3.3 Discussion

Microalgal cell walls present a significant energy barrier to algal product extraction and DNA introduction (Razon and Tan, 2011). This Chapter describes the attempts to determine cell wall composition and/or morphology through the use of various methods including; staining, light and confocal microscopy, enzyme screening, TEM, sonication and fungal digestion. Common laboratory staining practices proved unpredictable when used with microalgae, yet other methods yielded valuable information regarding cell wall structure and strength.

3.3.1 Algaenan is species specific and difficult to visualise

The inability for algaenan-containing strains to uptake stains also makes tracing changes in the wall difficult. Cell wall stains proved useful as an indicator of algaenan in green eukaryotic algae. The inability of some cyanobacteria to stain with cell wall stains such as calcofluor may be due to thick outer mucilaginous sheaths, which may have been devoid of β1,4 and β1,3-glucans. Cell wall staining was found to be too variable between and within species to be used as an indicator of cell wall changes after enzyme treatment. However calcofluor staining was further investigated for its potential as a cell wall mutant screen (Chapter 4.2.3).

Extensive TEM of all strains (Chapter 3.2.5) indicate that eukaryotic algae exhibiting poor staining with calcofluor or crystal violet likely contained the biopolymer algaenan, visible as a trilaminar structure (TLS). As for the cyanobacteria, TEM confirmed the presence of very thick gelatinous cell walls comprised of at least two layers. Due to the thickness and potentially complex (unknown) composition, this is likely to have hindered the efficacy of the stain. These microalgae and cyanobacteria also tended to show poor stain uptake with other stains. For example, DAPI (a nucleic acid stain) strongly stained the cell wall of some of these species. This could be due to the presence of some nucleic acids in the wall and/or poor penetration ability. BODIPY (a lipid probe) also showed staining of the cell wall in species with a thick cell wall. This is somewhat expected as lipids are known to play a strong part in the structure and function of the cell wall of many algae (Allard *et al.*, 2002).

Poor uptake of other cell stains is also noticeable in cyanobacteria with a thick wall. TEM observation of microalgal cell walls has been invaluable in visualizing differences in the cell walls between strains. A TLS could clearly be seen in the eukaryotic green strains

C. emersonii, *S. vacuolatus*, *P. ellipsoidea* and *C. saipanensis*, all of which showed marked differences in the structure of their cell walls (some with a single 'neat' algaenan layer, others with an amorphous TLS). This coupled with the lengthy TEM sample preparation would make it beneficial to find other means of describing cell wall differences in algae, in particular if the effect of culture conditions on cell wall morphology were to be investigated.

Thick walls of aplanospores of *H.pluvialis* give them low permeability, making chemical embedding, fixation and 'ultrathin sectioning' for electron microscopy almost impossible (Hagen *et al.*, 2002). This was noticeable when sectioning the filamentous cyanobacteria with thick cell walls. 'Pitting' could be seem when sectioning as the samples had become brittle and strength of the filaments pulled out from the resin slices and block.

3.3.2 Algaenan confers physical strength

Sonication experiments (to measure cell wall strength), when compared with the TEM images of the relevant strains, suggests that algaenan is mechanically very strong. *C. vulgaris* despite its relatively thick (and enzymatically resistant) cell wall and its small size was found to be most sensitive to sonication. Despite the larger cell size and very thin single-layered cell wall of algaenan, *P. ellipsoidea* is able to withstand much longer sonication than *C. vulgaris. S. vacuolatus* (possessing a thick cell wall and a single layer of algaenan) is second strongest to *C. emersonii* which has multiple algaenan layers atop a thick cell wall. The addition of SDS reduced the number of intact cells after sonication, most notable in *C. emersonii* where it reduces the number of intact cells by almost 30% compared to non-SDS values, for each exposure time tested.

It is important to note that altering a number of conditions may prove successful in breaking cells over a shorter time period. It would be interesting to alter amplitude, frequency and/or vessel size/shape to determine if cells could still be broken efficiently with a lower energy input. Concentration of cells to a slurry or paste could also alter the efficiency of sonication under different settings by altering the fluid dynamics of vibration and cavity formation and collapse (Santos *et al.*, 2009). Keeping the temperature low helps keep cavity collapse high energy and improves efficacy of sonication. Cooling of such small samples over the long sonication periods tested however, is not possible. It would be useful to determine the energy required to break one cell as a means of comparing cell strength, however energy losses from heat and sound, would make this value obsolete.

It was concluded that enzymes are not a suitable means of determining the composition of the microalgal cell wall due to its complexity and resistance to enzyme attack. Algae containing algaenan are highly resistant to enzymatic digestion. *C. vulgaris*, despite not containing algaenan still shows remarkable resistance to concentrated mixtures of commercial enzymes, suggesting a complex cell wall. However, the digestion protocol was based on a method by Hatano *et al.* (1992), which uses a buffer pH of 8. Many enzymes show activity between pH 6-8, yet some fungal enzymes show optimal activity between pH4-5 (Chaplin and Bucke, 1990). The activity of enzymes screened may have been largely dampened by the higher pH used.

3.3.3 Digestion of algaenan using Fusarium oxysporum f.sp. elaeidis

The FT-IR spectra of algaenan extracted from *C. emersonii* and *P. ellipsoidea* shows some similarity to that in published material. There were large losses of final product due to the number of steps required to extract algaenan, limiting the potential of the *F.oxysporum* digestion experiment. Macroalgae have been investigated as a substrate for ethanol production, suggesting there are fungi able to digest algal biomass (Horn *et al.*, 2000).

The rationale behind using a live fungus in the presence of algae (autoclaved, frozen and live) alongside extracts of algaenan (rather than exposing algae or algaenan to fungal extracts or culture supernatants), was to try and recreate circumstances under which fungi may use environmental cues to initiate production of particular enzymes. An extract of the organism could greatly vary depending on the conditions it is grown under. It is worth noting that even if a fungus is capable of digesting particular biological materials it may not utilise them as a carbon source.

Experiments were designed based on the limited quantity of algaenan extracted. This limited the number of experiments which could be conducted, ruled out the possibility of testing more fungi, within this project due to time constraints. Despite the fact that *F.oxysporum* f.sp. *elaeidis* did not appear to digest algaenan, the results could become valuable in the development of analytical techniques for algaenan digestion. For example, algaenan did not contain any contaminants detrimental to fungal growth (a concern due to the number of steps using strong chemicals in its extraction).

The faster growth rates and higher cell densities observed for *F.oxysporum* f.sp. *elaeidis* cultured with autoclaved algae (in both Cmin and C- media), may have been due to the difficulty in distinguishing algal cellular debris from fungal spores in these samples. Cell organelles, broken or deformed cells of algae had a similar appearance to some fungal spores and samples containing autoclaved algae contained distinctive sticky clumps of cells and cellular material. In these samples fungal cell counts were challenging and may appear significantly higher for this reason imparting a large error to the readings. Samples containing algaenan exhibited increased growth rates and cell densities, which could be due to some cell wall contaminants remaining in the algaenan (which appeared sticky and not completely homogenous). The visible quantities of algaenan added to samples also did not appear to change over the course of the experiments. From these observations it can be assumed that *F.oxysporum* f.sp. *elaeidis* cannot utilise algaenan as a carbon source, yet additional nutrients present in samples containing algaenan or algae can marginally supplement the growth of this fungal strain.

Although 'cell counts' for hyphal growth were estimated for samples containing hyphae, the growth may not be accurately represented. Hyphal growth may have resulted due to lowered carbon availability, or the presence of extracellular organic material (in samples containing algae) or even the small conical flask sizes used affecting mixing kinetics. Agar plate experiments despite being qualitative may still provide insight into changes in the behaviour of fungi in response to algaenan. Staining also proved ineffective for detecting any digestion of algaenan in algal cell walls or material. Extracted algaenan is therefore a preferred material for testing the efficacy of liquid fungal cultures to digest it. Due to the small quantities of algaenan extracted from large quantities of algal biomass, small experimental volumes would still be required for any future experiments using algaenan.

Long chain fatty acids, esters or alcohols were not detected in any of the samples analysed by GC-MS (data not presented). However two chemical signals identified as dedodecyl phthalate and squalene were detected in the GC-MS. Dedocecyl phthalate is likely a component of the cell wall (Chuck, 2011). Squalene (a terpene) is present in most organisms as a vital intermediate in the production of eukaryotic sterols (Spanova and Daum, 2011). Significant quantities from plant derivatives are found in olive oil and palm oil, yet the most abundant natural source is shark liver oil (Spanova and Daum, 2011). The increase in squalene from the controls suggests it is released into the media by *F.oxysporum* f.sp. *elaeidis*, rather than a

breakdown product of algaenan. GC-MS remains a very valuable tool in the analysis of algaenan breakdown.

Algaenan in *C. emersonii* comprises many layers within the cell wall, whereas *P. ellipsoidea* has a very simple layer of algaenan and an almost absent cell wall otherwise. Upon visual examination of the algaenan extracts *P. ellipsoidea* algaenan appears more homogenous than *C. emersonii* algaenan. As such *P. ellipsoidea* algaenan may contain fewer contaminants from the cell wall and could be more suitable for testing the ability of fungi to digest algaenan than *C. emersonii* algaenan. Other fungi known to digest tough biomaterials (such as *Fusarium solani*, *Ganoderma* sp., *Trichoderma* sp., or *Armillaria* sp.) must also be tested for their ability to digest the tough biopolymer. Naturally co-occurring organisms which may feed on algal cells (containing algaenan) could also be isolated from the environment for further investigation.

3.3.4 Further work

Ideally TEMs of the microalgae examined at JM should be repeated, not only to better cure samples (so as not brittle and easier to section) but to also view at a lower voltage to see more detail. An obvious variable to investigate is how culture conditions alter cell wall morphology, a well-known phenomenon in algae yet not thoroughly understood. However this would require extensive imaging for which TEM may not be suitable, which could restrict research to a few-algaenan free strains.

Sonication proved an effective method for measuring cell wall strength. However changing the frequency, vessel shape/size and amplitude could greatly reduce the energy input and as such would benefit from further investigation. It may be of interest to measure (using sonication) wall strength of cells that have experienced different growth conditions. This would reduce the number of samples, to ‘weaker walled’ growth conditions for TEM.

It would be beneficial to rescreen the enzymes, using a buffer of lower pH, ideally between pH 4-5. Enzymes could be further investigated as a means of breaking the wall or characterising it, yet rather than screening individual enzymes, natural ‘cocktails’ may provide great insight into the structure of algaenan or provide interesting enzymes for further study. These could then be split using protein chromatography to isolate the most effective

fractions for identification. This was out of scope for this project so as an alternative means to further investigate the cell wall, mutagenic methods were developed (Chapter 4).

3.3.5 Summary

The microalgal cell wall was found to be highly complex, physically strong and resistant to enzymatic degradation. Certain species exhibited algaenan in their outer cell walls, rendering their response to common laboratory stains unpredictable. TEM was shown to be very useful in visualising the cell wall of small 'unstainable cells'. Algaenan was shown to exist in many different arrangements in the cell wall and inferred a high degree of mechanical strength. Sonication proved a valuable method for measuring the strength of cell walls, yet requires further investigation for optimisation.

4. MUTAGENESIS OF MICROALGAE

4.1 Introduction

4.1.1 Benefits of genetic engineering

Modern agricultural food crops are the result of generations of selective breeding. For example, traditional (or 'wild') wheat has been bred to be shorter, increasing yield by selecting for those strain which divert their energy capture into grain (Kerridge, 1967). Starting strain selection is still very important, improvements in the use of traditional biofuel crops include the switch from 'C_3 plants' to 'C_4 plants', which have a higher potential for energy conversion as they accumulate more carbon by mass (Walker, 2009). The C_4 pathway evolved to increase CO_2 concentration proximal to RuBisCO in plants from hot and dry environments. Transgenics have successfully incorporated C_4 enzymes into a C_3 plant, resulting in the increased carboxylation efficiency of RuBisCO (RuBisCO is usually an inefficient enzyme, as it catalyses both oxygenation and carboxylation, resulting in the loss of 30-50% of assimilated carbon (Raines, 2006).

However this 'domestication' of crops is not easily applicable to algae, as most potentially profitable species lack a sexual cycle (Peng *et al.*, 2012). Also analyses suggest that natural selection of a beneficial trait for the individual cell (such as high chlorophyll content) conflict with the artificial selection of a desired trait (low chlorophyll content). GM strains are not only easier to generate but are believed to be more stable (Flynn *et al.,* 2010). Mathematical models regarding the generation of biofuel from algae show a potential 10 fold increase in productivity in GM versus unmodified algae (Flynn *et al.,* 2010). Algae could potentially become an invaluable model in industry and biological research, if there were readily available methods for genetic engineering. This way a prized strain could easily be tweaked to either improve productivity (lipid content), extractability (weakened cell wall), improve tolerance to conditions (productive at higher temperatures), improve flocculation (for harvesting) or even transfer a pathway that produces or secretes a valuable by-product. Biochemical pathway manipulation is believed to be imperative to scaled-up commercialisation of algal products (Purton and Stevens, 1997), as increasing knowledge of the pathways can help in regulating them or the interdependence of them (Scott *et al.*, 2010). One such example is the construction of the first artificial cell (Gibson *et al.,* 2010).

Recombinant DNA techniques to manipulate the genome have already been successfully applied in many industrial microorganisms (Campbell and Reece, 2002). Advances in the field of algal genomics however, have only occurred very recently with algal studies lagging far behind other eukaryotes (Walker *et al.,* 2005). Early successes with *Chlamydomonas reinhardtii* have catalysed the development of microalgal transformation systems in several other species (Walker *et al.,* 2005). Only in the last 10-15yrs have protocols been developed for a few lab models, increasing our understanding of the function and biology of these organisms (Walker *et al.,* 2005). Transformation of cyanobacteria is somewhat better established due to its bacterial nature, yet has only been described in a handful of species (Purton and Stevens, 1997).

There are requirements for the successful engineering of a microorganism: axenic clonal culture, established culture maintenance and single cell regeneration techniques, which could prove challenging for filamentous or multicellular species (Purton and Stevens, 1997). Available genome sequences are also important for classical genetic techniques (Guarneiri *et al.,* 2011). Complete sequences are only available for a few species: red alga *Cyanidioschyzonmerolae,* diatom *Thalassiosira pseudonoma* and green algae *Chlamydomonas reinhardtii* and *Ostreococcus tauri* (Walker *et al.,* 2005). Transcriptomics and proteomics are also dependent on genomic sequence data being available, lack of which has hindered this revived research area (Guarneiri *et al.,* 2011). Although *C.reinhardtii* is the most developed algal model to date, it is currently of little commercial importance, although it has provided a platform for the development of algal transformation technology (Purton and Stevens, 1997), such as selectable markers and reporter genes, vectors for foreign gene expression, RNAi gene silencing, DNA tagging, promoters and enhancer elements (Walker *et al.,* 2005) and microarray gene expression techniques (Eberhard *et al.,* 2006). The use of the transcriptome in some cases may bypass the need for genomic sequence data (Guarneiri *et al.,* 2011), yet for metabolic engineering, annotated genomes must be available (Wijffels and Barbosa, 2011).

One major challenge in algal engineering was the introduction of cloned DNA into the algal cell. There are a multitude of techniques available, yet due to the robust nature of the cell wall few of these methods are successful in the introduction of DNA into algae (Purton and Stevens, 1997). Direct introduction (microinjection or high velocity bombardment of DNA coated microparticles (‘biolistic process’)), and damaging the wall and allowing DNA to

diffuse inside (electroporation, agitation of cell wall deficient cells and glass beads or silicon carbide whiskers) have all had some success (Purton and Stevens, 1997). The glass bead method has been shown to be most successful in nuclear transformation rates, yet success depends on the use of protoplasts in *C.reinhardtii* (using purified autolysin or forced gametogenesis). Often these methods cannot easily be applied to strains which have limited genetic information and have not been studied (Purton and Stevens, 1997). These procedures may not be applicable to other algae as there can be significant differences in the complex cell wall between species and strains (Dunahay *et al.*, 1992).

Organelle transformation is somewhat easier and more stable. Currently *C.reinhardtii* is the only eukaryote where nuclear, chloroplast and mitochondrial genomes have been transformed, but has yet to be exploited. The chloroplast is involved in the TAG synthesis pathway (Figure 4.1) and due to the increased availability of chloroplast genomes (compared to nuclear), would be a good target for the engineering of other microalgae (Purton and Stevens, 1997).

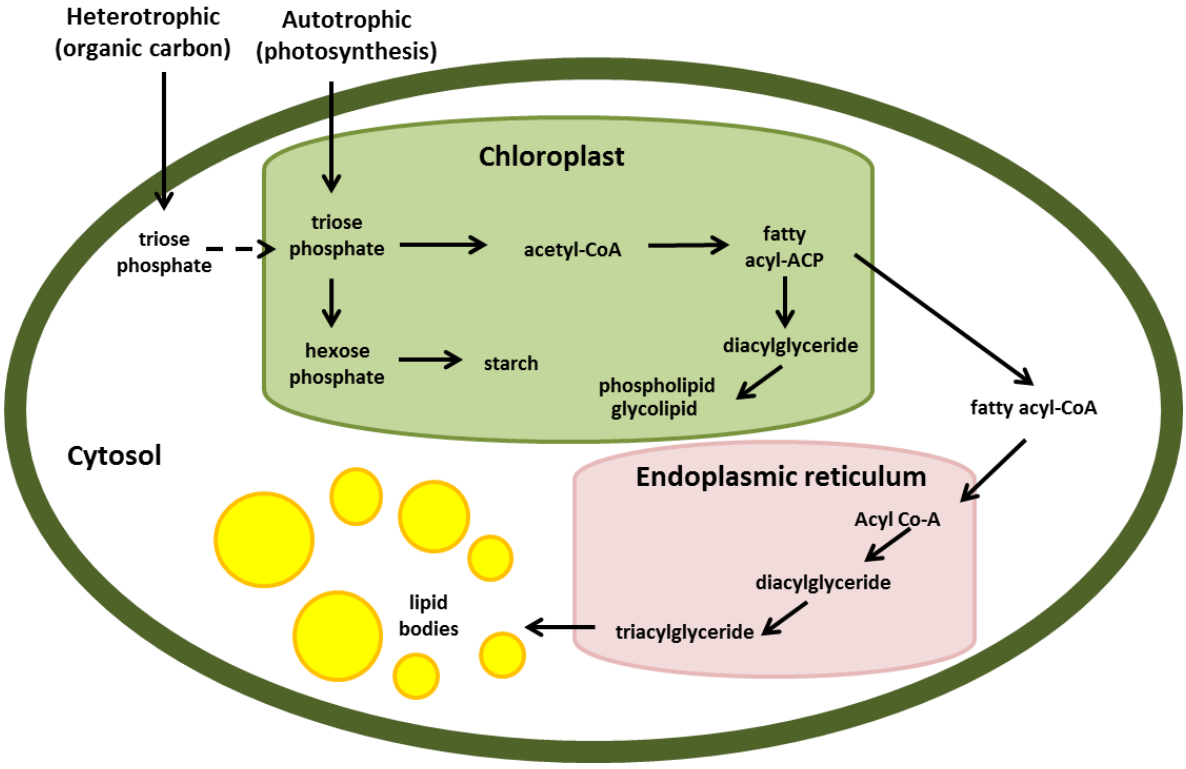


Figure 4.1: A proposed generalised pathway for synthesis of TAGs in a green microalga.
Adapted from, Scott *et al.*, (2010) and Radakovits *et al.*, (2010).

Rather than direct upregulation of the TAG pathway, a better strategy may be to shunt photosynthetic carbon from other metabolic processes. This technique led to a 10-fold increase in TAGs from a starch-less *Chlamydomonas* sp. UV mutant with an inactivated ADP-glucose phosphorylase pathway (Li *et al.,* 2010). It is much like the change in carbon metabolism seen in cells under nitrogen starvation, which switch from protein synthesis to lipid synthesis. However, there may be a 'ceiling' to what is achievable from the GM of algae in terms of productivity. To generate a high oil producing alga which also has a fast growth rate is the equivalent of a 'fat marathon runner' and may not be realistically possible (Montero *et al.,* 2011). Yet small changes can have a big impact on the commercial applications of an engineered strain. For example, *P.tricornutum* has undergone a 'trophic conversion' to be able to utilise glucose improving culture efficiency and giving it a wider application for clean-up (Walker *et al.,* 2005).

DNA markers are important to enable selection of transformed cells, usually a homologous gene which restores a mutant back to its WT phenotype (Walker *et al.,* 2005). This is straightforward when using a species which is haploid or has a haploid stage in their lifecycle (e.g. *C.reinhardtii* or *Scenedesmus* sp.), however, tends toward transient expression in species which are diploid or polyploid, such as *Chlorella* spp. as wild type alleles will lower transformant success rates and mask recessives (Walker *et al.,* 2005). Dominant gene markers are much more feasible for use with polyploids (Spicer, 2012). In many green algae (i.e. *Chlorella* spp. and *Scenedesmus* spp.) a cell may produce 2, 4, 8 or even 16 daughter cells depending on species and culture conditions (Mandalam and Palsson, 1997). Algal growth (G1 phase) requires light and to a lesser extent G2-M may also require photosynthesis (as a signal), meaning light:dark cycles can be used to control cell cycle stages. Good synchonicity may require 12/12h light dark cycles and is important when subjecting algae to any genetic modification (Otero and Goto, 2005)

Antibiotic or herbicidal resistance genes have been used successfully in the isolation of algal transformants (Walker *et al.,* 2005). However, several algal groups already have a natural resistance to common antibiotics (Purton and Stevens, 1997). Should algal genetic 'tailoring' become successful herbicide or antibiotic sensitivity would be necessary to introduce to increase 'end product partitioning' (Wilkie *et al.,* 2011). Algal transformants are often in low yield and show high reversion rates or show unstable expression (Purton and Stevens, 1997). Inappropriate codon usage has been thought to be a cause. Successful foreign genes have

become integrated by selecting genes with a similar codon bias to endogenous genes (high GC content) (Walker *et al.,* 2005). Many algal transformants have markers introduced at seemingly random loci (non-homologous recombination). This random integration disrupts functionally important genes and although many phenotypes have been generated this way and are of interest to study, where metabolism has been negatively affected, they are not useful commercially (Purton and Stevens, 1997).

There is a need for a more robust genetic approach to microalgal modification with generic tools and a simple high throughput approach that does not rely on prior sequencing. A successful method in one species is not necessarily easily transferrable to another even closely related alga (Purton, 2011). It needs to be applicable to multiple known strains and uncharacterised ones, to improve scope for industrial use of more strains (Spicer, 2012). Diatoms can fix large quantities of CO_2 and their intricate silicon frustules make them a desirable candidate for GM to create 3D structures for nanotechnology for example (Walker *et al.,* 2005).

Currently the development of algal genetic research is rife, for example the 'Phycobrick' project, at University College London, where a selection of compatible genetic 'chunks' are being engineered in order to provide a 'toolbox' for fine tuning of exogenous DNA targeted to particular species (Purton, 2011). The relatively new company 'SpicerBiotech' is developing a platform for the custom GM of algae, using methods that work for different groups of algae (Spicer, 2012). These methods rely on electroporation (higher electric field 10-11kV, multiple pulses, high cell density), and the use of cheap negative selection markers or algae sensitive to the techniques (Spicer 2012).

4.1.2 Mutagenesis

Mutagenesis is still a popular tool for generating interesting phenotypes to better understand gene function (Comai and Henikoff, 2006). Mutagenesis is suitable for the study of any organism for which genomic resources are limited, which is highly applicable to microalgal research (Walker *et al.,* 2005). Mutagenesis has been extensively applied in the study of plants in the form of TILLING ('targeting induced, local lesions in genomes') lines, which provide a range of mutant alleles for study (Comai and Henikoff, 2006). Mutation detection falls into two classes, screening for presence of a known mutation (with PCR primer) or mutation discovery. The latter is more challenging, with a high rate of false positives (Comai

and Henikoff, 2006). TILLING minimizes efforts required to find mutations but does not help with 'ascertaining the resulting phenotype' (McCallum *et al.,* 2000).

Mutagenesis can be used to help ascribe gene function in well described species such as *Chlamydomonas* spp. (Polle *et al.,* 1999) using a systemic approach (Melis, 2009). *Haematococcus pluvialis* unlike *Chlamydomonas* spp. has a more plant-like cell wall (containing cellulose) and is therefore a good model for cell wall biology. Mutants have helped give insight into genetic regulation within givn species. For example, cell wall mutants of *H.pluvialis* show down-regulation of certain proteins compensated for by overexpression of other proteins (Wang *et al.,* 2005). Mutagenesis is a particularly useful tool for *Chlorella* spp. (an important oleaginaceous genus with a high CO_2 fixation rate) from which many interesting strains have been generated, for example, a temperature tolerant strain (Ong *et al.,* 2010).

Mutations are largely random and can be induced using chemicals (e.g. EMS or MNNG) or radiation, X-rays or UV (McCallum *et al.,* 2000), (Table 4.1).

Table 4.1: Physical and chemical mutagens and the mechanism of mutation
Adapted from Montelone (1998).

Mutagen	Types of mutation	N.B.
Physical		
X-rays	α-particles cause double strand DNA breakages.	Mutagenic, teratogenic, and carcinogenic. Sources are not always available.
UV	Causes dimerization of adjacent pyrimidines, resulting in distortion of DNA structure and replication errors.	Readily available in the form of biocidal radiation lamps in many laboratories.
Chemical		
Ethyl methanesulfonate (EMS, $C_3H_8O_3S$)	EMS induces random point mutations by nucleotide substitution (guanine alkylation). During replication thymine, instead of cytosine, is placed opposite alkylated guanines. Following subsequent replications, the original G:C base pair becomes an A:T pair.	Mutagenic, teratogenic, and possibly carcinogenic
Methylnitronitrosoguanidine (MNNG, $C_2H_5N_5O_3$)		MNNG is not readily available and is highly mutagenic and teratogenic.

UV irradiation is known to affect many metabolic processes in algal systems, however it typically has low mutant yields among survivors (Lambert *et al.,* 1980). Although EMS is a widely used mutagen in the biosciences, there is little published information on how it affects algae (Tripathi *et al.,* 2001). MNNG is no longer widely available (data not shown). When

transforming or mutagenizing algae it is important to use healthy (exponential growth phase) algal cells, as under these conditions ploidy of 4-8C is observed in *Chlorella* sp. Under nitrogen or stress conditions however, transient higher ploidy (8-12C) has been observed (Kobayashi *et al.,* 1987). Ploidy can also affect how often a strain can undergo mutagenesis. Serial mutation is only suitable for haploid species, and difficult to do in diploids or polyploids (Kobayashi *et al.,* 1987). Most microalgae have no sexual cycle, therefore cannot be backcrossed to outcross detrimental mutations in an otherwise desirable mutant (Schlarb-Ridley, 2012).

Throughout their evolutionary history algae have been exposed to oxidative and radical stresses and as a result have developed very effective protective and reparative mechanisms (Pulz and Gross, 2004) and produce antioxidants and detoxifying enzymes (Pattanaik *et al.,* 2008). As a result, successfully mutated strains usually exhibit lower fitness and can only be modified to an extent, before other aspects of cellular metabolism are negatively affected (Pulz and Gross, 2004). Hence it is important to determine the presence of other undesirable side effects (Melis, 2009). Additionally algae have low phenotypic stability for WT mutants and GM. Long-term preservation of a certain strain trait cannot be guaranteed. The only viable way is to cryopreserve but this is not always suitable or possible with some strains (Day *et al.*, 2012).

4.1.3 Selection of mutants

Essential to the success of mutagenesis in creating novel strains, is its combination with an efficient high throughput screening method for the detection of desirable phenotypes (Sandesh Kamath *et al.,* 2008). The simplest screening method is to plate mutagenized cultures and visually observe colonies for any abnormalities (Ishikawa *et al.,* 2004). For algae where techniques are more established (i.e. *Chlamydomonas reinhardtii*) more sophisticated methods of screening can be used (McCarthy *et al.,* 2004).

Photoinhibition and self-shading of chlorophyll-rich algae prompted the generation of low chlorophyll strains with truncated light-harvesting systems, in particular for the cultivation of oleaginaceous species such as *Chlorella* sp. (Polle *et al.,* 2002). This phenotype is easily spotted when scanning colonies for a difference in pigmentation, as low chlorophyll mutants tend to appear yellow-green to the eye (Melis, 2009). Some mutants have the same growth rates in light as normal strains, whereas others have greatly altered metabolism and may

compensate by increasing their heterotrophic efficiency (Urano and Fuji, 2000). Colour mutants often show strong reversion (Urano and Fuji, 2000).

The use of a fluorescence-activated cell sorter (FACS) can enable high throughput screening by selecting for individuals with low autofluorescence (Melis, 2009). The same method can be applied to any single celled alga which could be differentiated using the FACS (Montero *et al.,* 2011). However, in order to visualise phenotypes in this way, stains are often employed, which tend to exhibit unusual behaviour when used with algae (Chapter 3.2.2). For example, nile red staining is a semi-quantitative method useful for screening for hyper-lipid producing strains (Gao *et al.*, 2008). The success of nile red staining on oleaginaceous microalgae varies significantly between different species and often requires the addition of large quantities of DMSO (20-30%) and elevated temperatures (40°C) to aid permeation through the cell walls (Cooper *et al.*, 2010). High levels of DMSO are lethal to algae and as a result, many oleaginaceous species cannot be selected for using this method of visualisation, making rapid screening difficult. DMSO concentrations required for the lipid stain BODIPY are lower than that for nile red (0.02-2%), with algal cells remaining viable (Cooper *et al.*, 2010). However, BODIPY also strongly stains glycolipids in the cell wall and can give false positives depending on the species used and as such, may be suitable for the selection of cell wall mutants (data not shown).

Separating and extracting oil from algae comes at a high cost (Razon and Tan, 2011). It is therefore of interest to modify cell walls to make extraction easier (Purton, 2011). Cell wall defective strains of algae also increase the success of DNA transformation protocols (Fuentes and Vanwinkle-Swift, 2003). As the cell walls of microalgae are so variable and understudied (Wang *et al.,* 2005), mutagenesis is ideal for producing interesting phenotypes to study. However as discussed in the previous Chapter (3.2.2) staining of the cell wall is not straightforward, which poses a challenge when it comes to screening.

A cell wall-deficient mutant of *C.reinhardtii* was found to show significantly lower light scattering compared to the wild type when measured spectrophotometrically (Svensen *et al.,* 2007). If this phenomenon is also true for other species (and this change in light scattering could be detected by FACS), light scattering properties could become another high throughput method for selecting for cell wall mutants within a population of mutagenized cells. Cell wall mutants can be identified by colony morphology on agar, with colonies showing swollen,

amorphous cells with irregular edges (cell wall defects) (Fuentes and Vanwinkle-Swift, 2003). In 1976, Loppes created cell wall mutants of *C.reinhardtii*, which grew well on solid media (where vital metabolic constituents would leak and remain close to cells) but exhibited no growth in liquid media, where leakage would dilute metabolites. Many cell wall defective strains also show increased lysis (Fuentes and Vanwinkle-Swift, 2003). As a result, a screening method for viable cell wall mutants, should be conducted in liquid media. It will be important that strains do not just have a greatly weakened cell wall, it must still have some mechanical strength to withstand shear forces present in culture systems. Cell walls do not necessarily need to be weaker or thinner, but perhaps less complex and easier to break when desired.

The commercial use of a genetically modified organism (GMO) is not only stigmatised but also tightly regulated (Walker *et al.,* 2005). GM crops for example are cited as a potential threat due to the difficulty of controlling the 'escape' of genetically modified material, such as pollen. Although microalgae do not form pollen, they could spread rapidly through water systems and air aided by weathering and transfer from animals such as waterbirds (Pulz and Gross, 2004). Large scale culturing will therefore need to be enclosed by law, and have procedures in place to prevent escape, which will restrict GM algae to closed systems (Walker *et al.,* 2005). It is therefore of great importance to state that the genetic manipulation of algae must 'complement and not substitute' the screening of new species (Pulz and Gross, 2004).

4.2 Results

4.2.1 EMS mutagenesis

Ethylmethane sulfonate (EMS) was primarily investigated as a mutagen for use with our microalgal strains, due to the higher incidence of mutants which should be yielded compared to UV mutagenesis (Lambert *et al.*, 1980). Fast growing asexual oleaginaceous haploid strains of *Chlorella vulgaris* and *Pseudochoricystis ellipsoidea* strains 'obi' and 'ni' (capable of accumulating up to 82wt% lipids (Satoh *et al.*, 2011)) were chosen to optimise the mutagenic protocol. MNNG was not used (not only due to its unavailability) because it typically induces large multiple and linked mutations, which in a haploid organism is more likely to be fatal than produce mutants. Using a protocol from McCann (2009) as a guide, 'optimal mutagenic dosages' equate to ~10% survival. This required the construction of calibration curves for each different type of mutagen and different species. For example, McCann, (2009) found that diploid *Chlamydomonas reinhardtii* required a significantly higher mutagenic dose (40min exposure) than haploid *C.reinhardtii* cells (13min exposure) to reach 10% survival.

EMS mutagenesis was found to give very variable results with each replicate experiment for *C. vulgaris* and *P. ellipsoidea* (Figure 4.2 and 4.3). Within each experiment there is also variation in cell survival (no replicates carried out for each individual date).

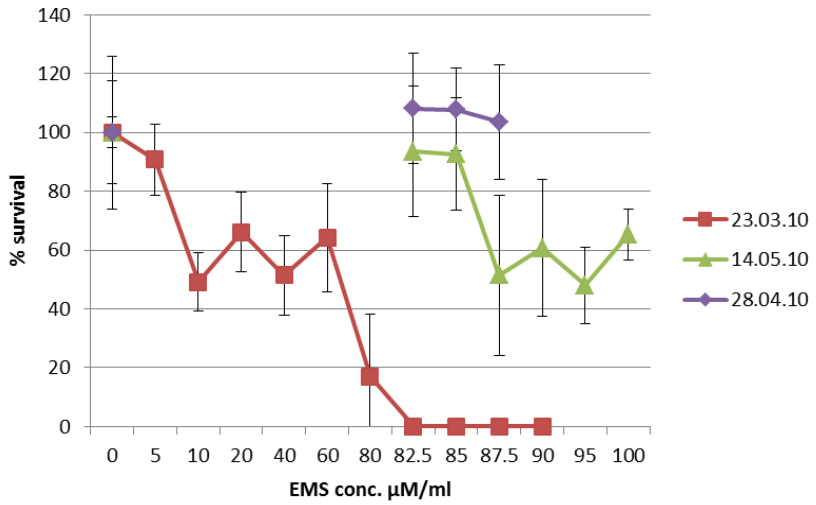


Figure 4.2: Replicates of EMS mutagenesis calibration curves for *C. vulgaris*, using various concentrations of EMS over a10min exposure.
Survival values (colony counts) are given as a percentage compared to the control from plated aliquots of mutagenized culture. For each concentration ten replicate agar plates were prepared, error bars = S.D. Each series was intened as a repeat experiment.

P. ellipsoidea ‘obi’ and ‘ni’ behaved similarly in response to EMS but required a significantly higher concentration than *C. vulgaris* until an effect was observed (Figure 4.3). At these concentrations EMS no longer mixed well with the culture but began to separate into phases. To remedy this exposure time could be lengthened. *P. ellipsoidea* when in the exponential growth phase (necessary for mutagenic treatment) forms clusters (data not shown). This may have caused mutagenized cells to stick to healthy cells, which during plating would have formed more ‘viable’ colonies, which may account for the observed higher survival rates. This would make it more difficult to isolate colonies derived from single cells.

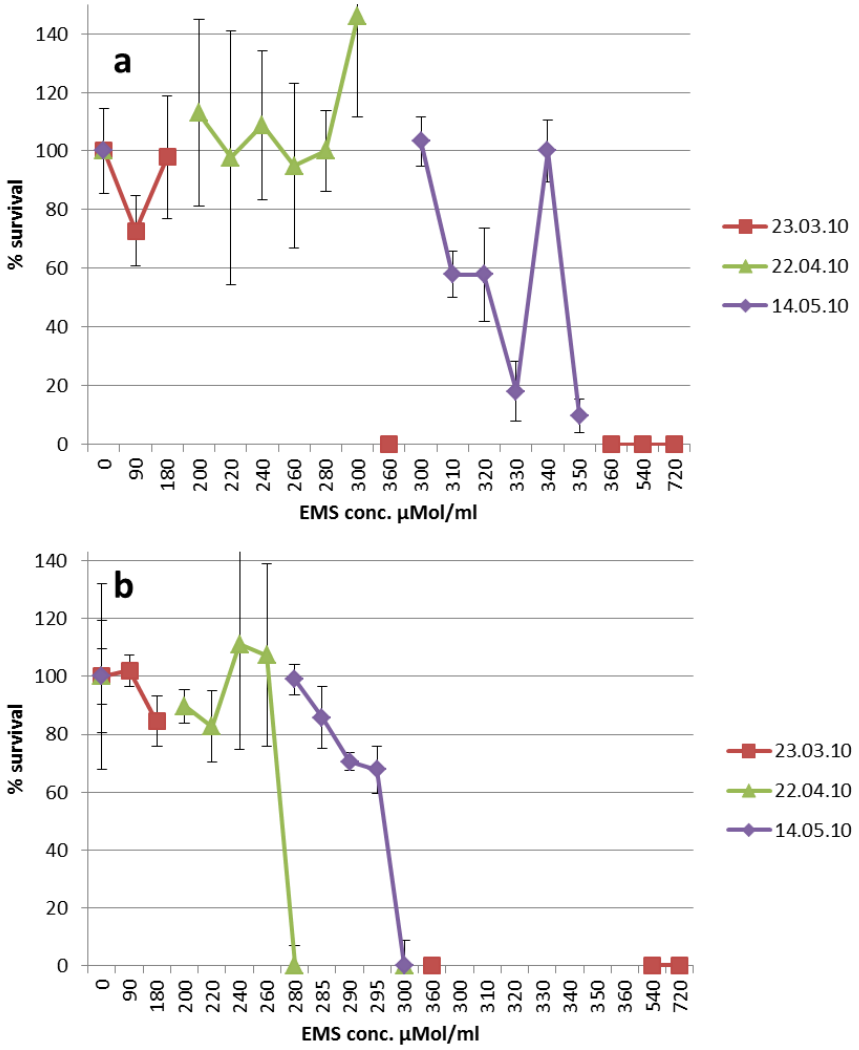


Figure 4.3: EMS mutagenesis calibration curves for *P. ellipsoidea* (a) ‘obi’ and (b) ‘ni’, using various concentrations of EMS over a10min exposure.
Survival values (colony counts) are given as a percentage compared to the control from plated aliquots of mutagenized culture. For each concentration five replicate agar plates were prepared, error bars = S.D. Each series was intened as a repeat experiment.

The decrease in efficacy of EMS as a function of cell survival could be explained by the degradation of EMS. Due to the variability in results, fluctuation within individual experiments and higher mutagenic doses, another means of mutagenizing was developed.

4.2.2 UV mutagenesis

UV mutagenesis was investigated as a means of mutagenizing our microalgae due to the variability in cell survival when constructing calibration curves for EMS mutagenesis. UV mutagenesis induces low level mutations (by distorting DNA as a result of dimerization of adjacent thiamine base pairs) and was therefore suitable for haploid species *C. vulgaris, C. emersonii, S. vacuolatus* and *P. ellipsoidea*. UV mutagenesis was observed to produce less erratic results than EMS (Figure 4.4). Results were also repeatable with *C. vulgaris* with a 10% survival rate at 10min. *C. emersonii* and *S. vacuolatus* required a higher dose of UV to reach 10% survival, which may be conferred by the layer of algaenan in the outer cell wall. Algaenan could also potentially act as a protective screen for UV, as excretion of particular compounds to the outer regions of the cell wall for UV protection is a well-documented observation in cyanobacteria (Pattanaik *et al.*, 2008). UV mutagenesis of *P. ellipsoidea* (as per EMS mutagenesis) required a higher dosage of mutagen (24min) than *C. vulgaris* (10min) to reach 10% survival (Figure 4.5).

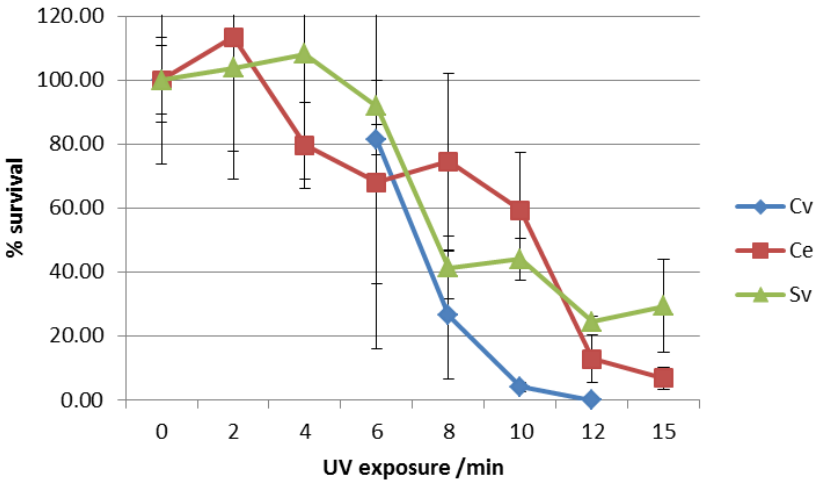


Figure 4.4: UV mutagenesis calibration curves for *C. vulgaris, C. emersonii* and *S. vacuolatus,* over different exposure periods.
Survival values (colony counts) are given as a percentage compared to the control from plated aliquots of mutagenized culture. Three replicates of each were performed, error bars = S.D.

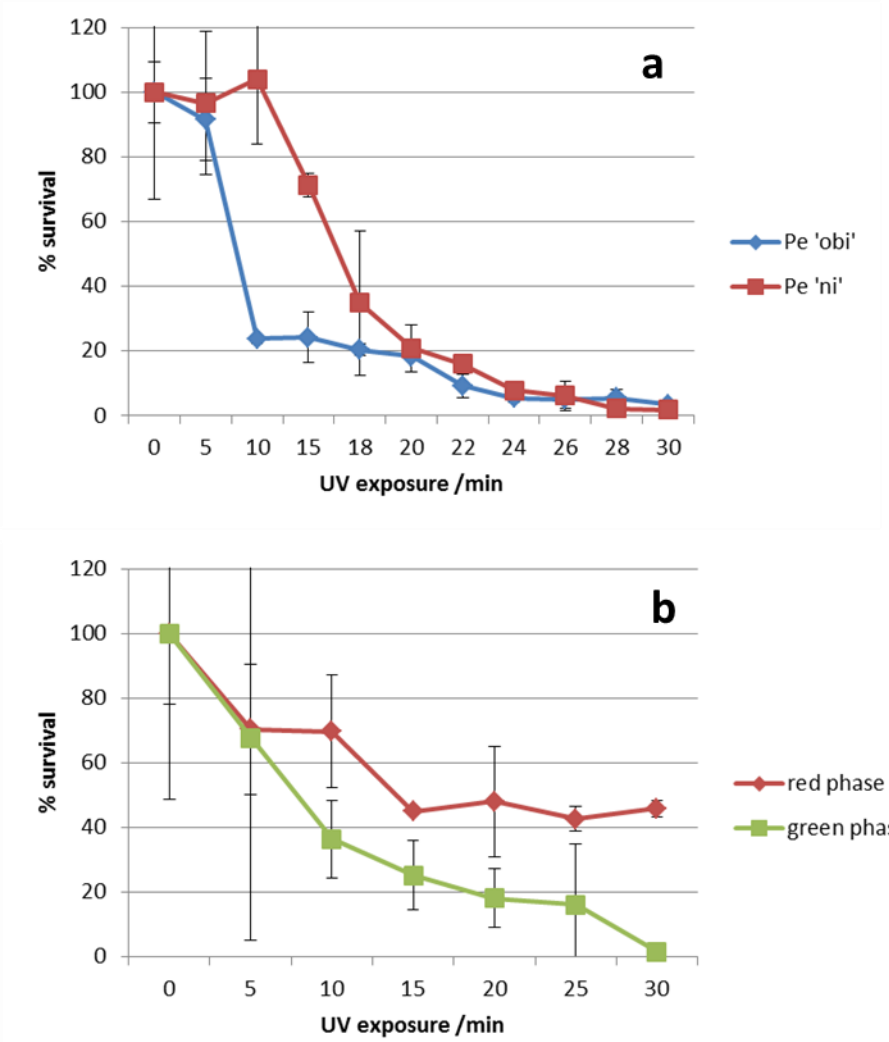


Figure 4.5: UV mutagenesis calibration curves for (a) *P. ellipsoidea* 'obi' and 'ni' and (b) *H.pluvialis* over different exposure periods.
Survival values (colony counts) are given as a percentage compared to the control from plated aliquots of mutagenized culture. Three replicates of each were performed, error bars = S.D.

Haematococcus pluvialis was also tested. Its cell wall morphology changes dramatically between the green (motile, growing) phase and the red (cyst, stressed) phase. In the red phase *H.pluvialis* forms a very thick cell wall, almost 2-3 times thicker than the green phase. The UV mutagenesis calibration for *H.pluvialis* showed that red phase cells required a much higher dose of UV, again likely conferred by the thicker cell wall.

The success in determining a replicable 10% mutagenic dose for *Chlorella* strains, allowed generation of a mutagenized cell library. Due to the higher oil content and larger cell size, efforts were focused on *C. emersonii* for the generation of a cell wall mutant. 100 plates were prepared each carrying approximately 100 colonies.

4.2.3 Screening for mutants

The simplest screening method is to plate mutagenized cultures and visually observe colonies by eye for any abnormalities (Ishikawa *et al.*, 2004). The mutagenized library of *C. emersonii* was screened for interesting phenotypes under the inverted microscope. Cell wall mutants can be identified by colony morphology on agar. Swollen, amorphous cells with irregular edges, usually indicate cell wall defects (Fuentes and Vanwinkle-Swift, 2003).

Screening colonies revealed that most colonies appeared visually identical to controls (Figure 4.6a). Two mutagenized colonies appeared different compared to the control. A yellow-green 'pale colony' (Figure 4.6b, termed pale mutant (PM)) displayed growth comparable to wildtype (similar colony size) and cells appeared approximately 1.5 times larger. Another small colony (which exhibited very poor growth) also showed irregular cell morphology, and may have been a cell wall mutant (Figure 4.6c).

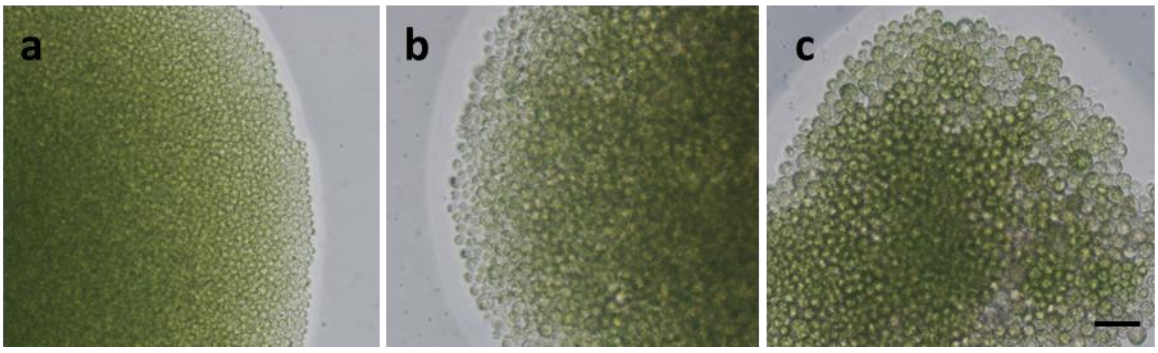


Figure 4.6: Inverted microscope images of colonies derived from UV mutagenized cells of *C. emersonii.*
(a) wild type (WT), (b) pale mutant (PM) and (c) potential cell wall mutant. Bar = 20µm.

The cell wall mutant, did not grow when transferred into liquid media, something which has been observed in other cell wall mutants in literature, too 'leaky' to survive in liquid culture (Loppes, 1976). The pale mutant however, appeared to grow well in BBM and retained its pale yellow-green colour (no or very slow reversion). This mutant was subjected to further analysis. Upon observation with the light microscope, PM looked similar to a photosynthetic mutant of the *C.reinhardtii* tla1-CW, (pale and speckled inside) (Pilon *et al.*, 2011).

In an attempt to screen *C. emersonii* mutants more efficiently and rapidly, it was decided to develop an assay involving fluorescence activated cell sorting (FACS) and calcofluor. The inability for cell walls of algaenan-containing species (such as *C. emersonii*) to stain with calcofluor, made it an excellent marker for mutants devoid of algaenan. Prior to cell sorting

C. emersonii underwent toxicity testing of calcofluor, to ensure cells could survive staining to enable sorting of viable cells based on calcofluor fluorescence. Other haploid strains were also tested, in the event they should be mutagenized and subjected to screening in the future (Figure 4.7).

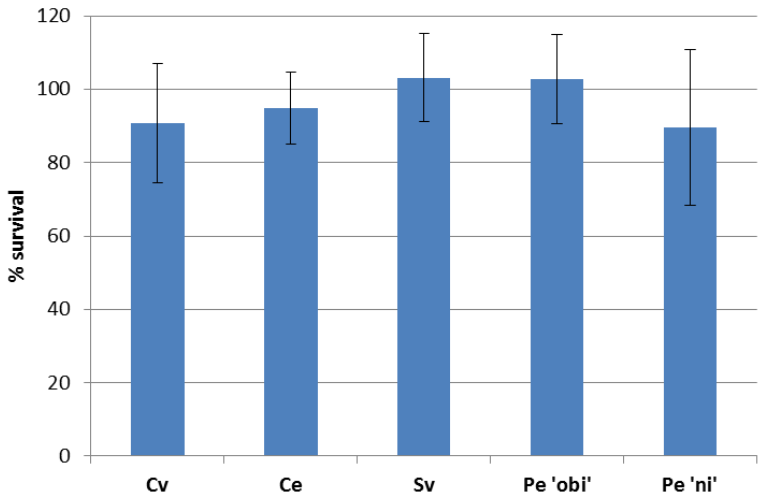


Figure 4.7: Percentage survival of *C. vulgaris, C. emersonii, S. vacuolatus* and *P. ellipsoidea* 'obi' and 'ni' after calcofluor staining compared to a control.

No major sensitivity to treatment with calcofluor was observed for any of the algae, with all strains having a survival rate of >90% (Figure 4.7). This could be explained by the inability of calcofluor to bind to cells containing algaenan. However *C. vulgaris* (which stains strongly with calcofluor) also survives treatment so this would suggest a low toxicity of calcofluor despite successful binding to the cell wall. This deemed it suitable as a screen for viable cells lacking algaenan.

FACS was investigated as a high-throughput method for the selection of cell wall mutants, due to the low yield nature of mutagenizing microalgae (Lambert *et al.*, 1980). The main premise is to mutagenize a culture, allow cells to undergo a few rounds of replication (so as not to stress 'freshly' mutagenized cultures) prior to staining, followed by staining with calcofluor to enable sorting using FACS (for chlorophyll positive ('healthy' cells) and calcofluor positive cells) (Figure 4.8). Cells are then allowed to undergo a few more rounds of replication, before staining and assessing the difference in level of staining of a mutagenized and sorted culture versus a WT culture. If algaenan-deficient mutants have been yielded, the final culture should show a higher percentage of stained viable cells than the WT. If algaenan deficient mutants are found, cell wall strength can be measured (using sonication) and

observed with TEM and compared to WT. However, it is important to reiterate that mutants should possess an altered or simplified cell wall, not necessarily weaker, as cells must still be capable of withstanding shear forces when mixed in culture.

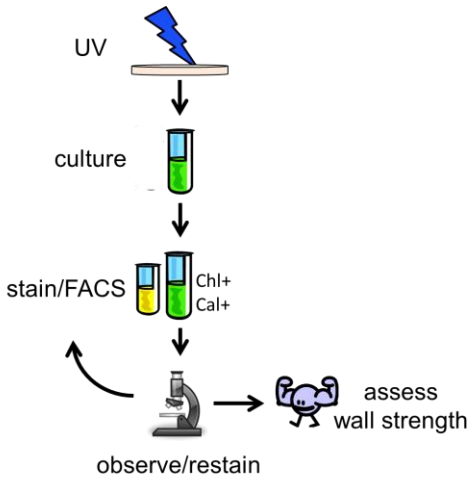


Figure 4.8: Schematic outlining a proposed high-throughput assay for the selection of cell wall mutants, using FACS and calcofluor staining of the cell wall.
C. emersonii contains a stain-resistant biopolymer (algaenan) in its outer wall, which infers significant mechanical strength. Staining of a mutagenized cell may indicate an algaenan-free mutant, which could be isolated using FACS.

The first round of selection selected for cells of a healthy size and chlorophyll fluorescence with additional calcofluor fluorescence (indicators of a potential algaenan-free mutant) (Figure 4.9). Selected cells (Figure 4.9b) grew at a rate comparable to wildtype in liquid media, yet upon restaining comprised phenotypically WT cells with no calcofluor staining (suggesting the presence of algaenan). This suggests a high level of false positives and the need to re-sort and/or re-mutagenize cells. Several rounds of mutagenesis however would result in low yields of healthy mutant cells due to the accumulation of mutations. Hence it would be best to simply subject a once-mutagenized culture to several rounds of sorting with FACs to eliminate false positives cells.

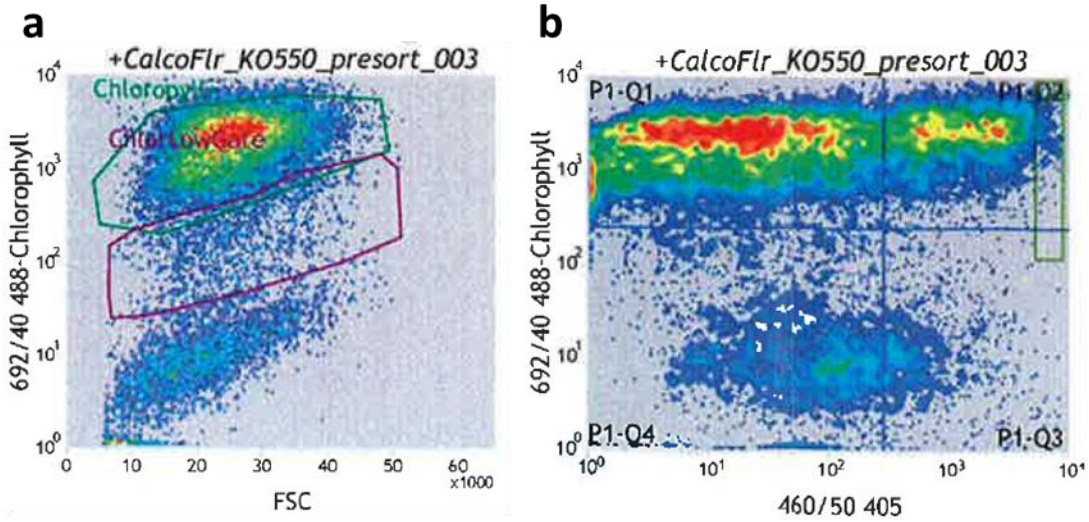


Figure 4.9: FACS scatter-plots gating for (a) chlorophyll fluorescence and cell size ('FSC') and (b) chlorophyll fluorescence and calcofluor fluorescence ('460/50 405').
'Gating' is denoted by the green and purple boxes and allows for selection of a sub-population from within the sample. In (b), the narrow green box indicates the gate used to select for a chlorophyll and calcofluor fluorescent sub-population, which may be indicative of a cell wall mutant. FSC = 'forward scatter' a measurement of particle (cell) size.

4.2.4 Pale mutant characterisation

The pale mutant (PM) of *C. emersonii* (yielded using UV mutagenesis) was subjected to further analysis, in case it exhibited any desirable features such as tolerance to particular culture conditions, increased lipid content or a preferable FAME profile. Firstly WT and PM strains were plated on BBM agar plates with 0.1% added acetate and cultured under different light conditions (Figure 4.10). Pale mutants are desired in PBR culture systems, where self-shading of chlorophyll-rich cells can lower productivity (Polle *et al.*, 2002). Pale mutants however can vary greatly in their photosynthetic metabolism and may compensate by being more efficient at heterotrophic growth.

Figure 4.10: Effect of light on growth of WT and PM strains of *C. emersonii* on BBM 0.1% acetate agar plates.
Dark-light = 0, 50,100,200 µMol photons m^{-2} s^{-1}.

In the dark PM appeared to be more efficient than WT at utilising acetate in the medium for growth (Figure 4.10). Under high light WT appeared to grow more vigorously. A simple liquid medium experiment was carried out to compare growth of WT and PM under 'standard conditions' (Figure 4.11). PM had an 'adjustment period' before showing exponential growth in light compared to WT, but did begin to catch up at day 15. From day 17, WT overtook PM in cell count but optical density (OD) did not dramatically change. As OD is a good indicator of biomass, this suggests a reduction in cell size of PM as the culture ages. Biomass measurements were not taken for these experiments as previously sampling small quantities gave very variable results (Chapter 3.2.1). A large quantity of biomass would have had to have been sacrificed and maximal biomass was needed for extraction and analysis of products.

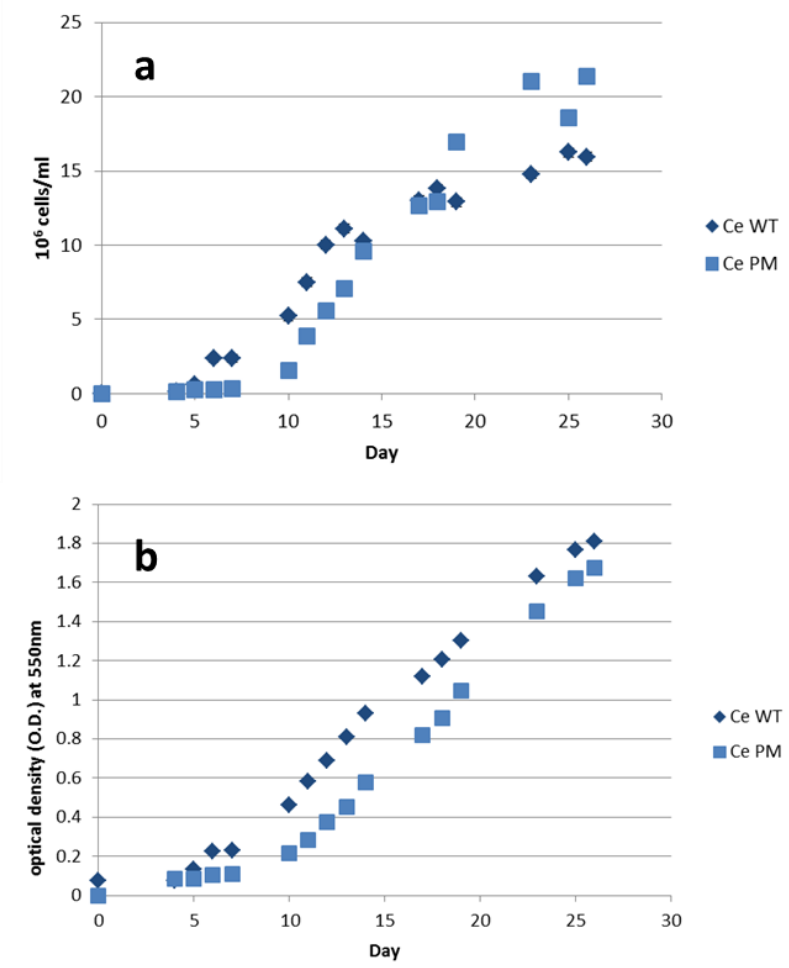


Figure 4.11: Growth comparison between PM and WT *C. emersonii* in liquid cultures under 'standard' culture conditions, using (a) cell count and (b) OD at 550nm.
Repeats were made for each measurement. Error bars = S.D.

In order to more closely examine growth characteristics under different light and media conditions, both PM and WT *C. emersonii* were cultured in standard BBM and BBM with added acetate (as a carbon source) and half of each culture subjected to a period of nitrogen starvation after 14d. Each of these media conditions was also examined under different light conditions (0, 50, 100, 200µMol photons m^{-2} s^{-1}, dark, low, medium, high).

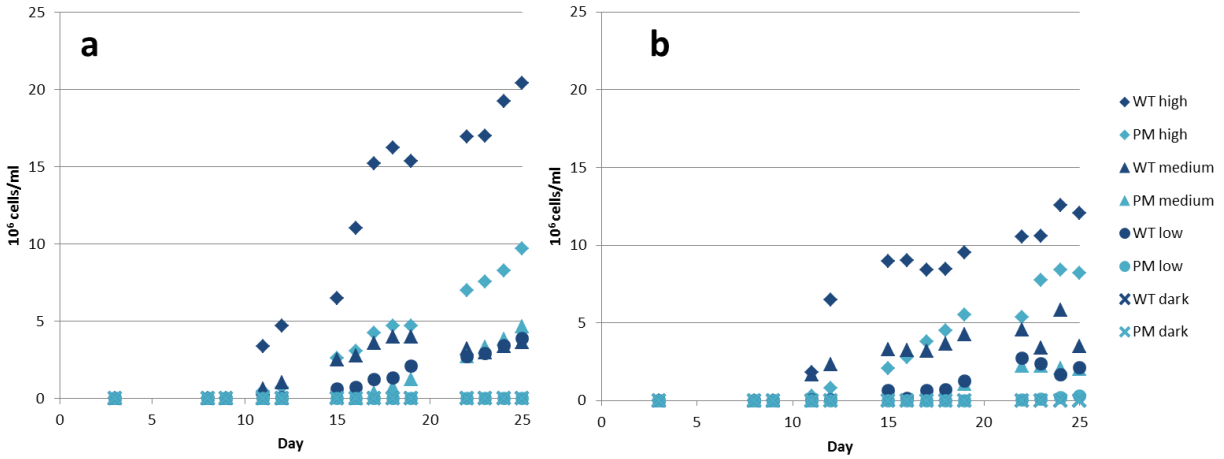


Figure 4.12: Growth characteristics of PM and WT *C. emersonii* under different light conditions.
(a) BBM and (b) BBM with nitrogen starvation after 14 days (BBM N-). No repeats were performed due to spatial constraints.

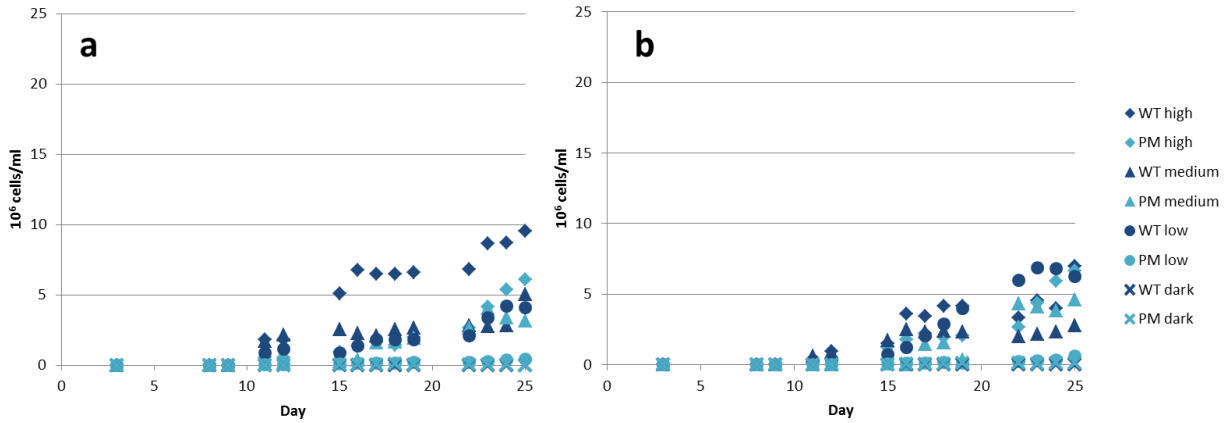


Figure 4.13: Growth characteristics of PM and WT *C. emersonii* under different light conditions.
(a) carbon supplemented medium (BBM C+) and carbon supplemented medium with nitrogen starvation (b) (BBM C+ N-). No repeats were performed due to spatial constraints.

In standard BBM culture medium at low light PM and WT growth was comparable, yet at high light WT exhibited faster growth. For carbon supplemented BBM (BBM C+) WT growth is impaired compared to non-carbon supplemented media under all light conditions. PM growth was also slightly impaired but not as strongly as WT. Under high light and carbon supplementation WT was still more productive than PM overall. Both WT and PM cells grew larger in carbon supplemented (C+) conditions, so despite the lower cell count

biomass may not be affected (data not shown). Under all media conditions those cultured under the highest light consistently had the highest growth rates (Figures 4.12 and 4.13).

The experiment was repeated for the 'high light' conditions (~200µMol m^{-2} s^{-1}) in order to investigate the effect of these conditions on FAME production between WT and PM. Final samples were lyophilised and FAMEs extracted using the 'beadbeater method' outlined in Chapter 2.2.28.

Interesting mutants should be screened for other phenotypic traits, including the ability to produce and accumulate high concentrations of specific compounds (fatty acids and/or carotenoids), nutrient uptake potential, and/or greater tolerance to a range of environmental and culture conditions (light intensity, temperature, CO_2 concentration, salinity or pH). For that reason, all media experiments for WT and PM were repeated under the high light conditions (yielding highest growth rates for both strains) and subjected to lipid extraction and transesterification with resultant FAMEs analysed to ascertain if there are any differences in metabolism between the strains, and if either strain or culture condition increased suitability of a strain for biofuel production.

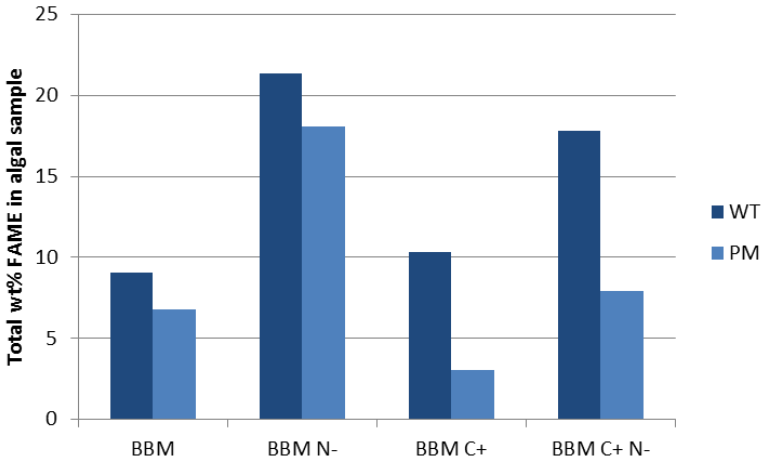


Figure 4.14: Total FAME content as wt% of WT and PM *C. emersonii* cultured in different media. Cultured under 'high light'(200µMol photons m^{-2} s^{-1}) conditions. No repeats were performed due to spatial constraints.

In all cases nitrogen starvation increased lipid content. WT consistently accumulated a higher quantity of FAME than PM (Figure 4.14). Carbon supplementation increased FAME content in WT if not nitrogen starved. Yet carbon supplementation during nitrogen starvation caused a reduction in lipid content. PM accumulated half the lipid in the presence of carbon under

nitrogen sufficient conditions, unlike WT, which accumulated more lipid (twice the quantity compared to PM).

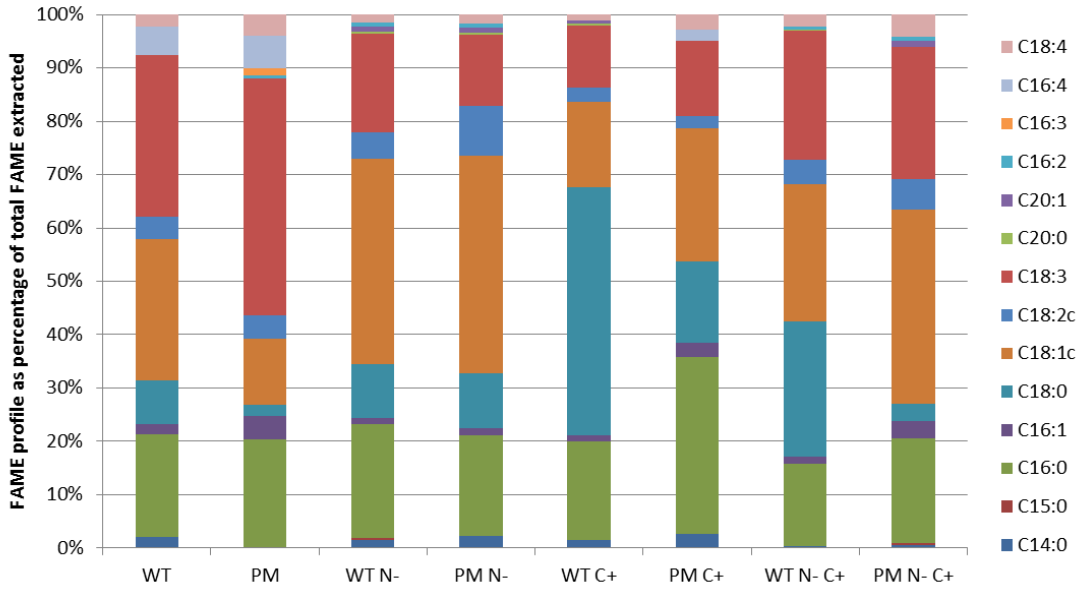


Figure 4.15: FAME profiles for WT and PM under different growth conditions, as a percentage of total FAME content.
Cultured under 'high light'(200µMol photons m^{-2} s^{-1}) conditions. No repeats were performed due to spatial constraints.
#

FAME profiles between PM and WT for all nutrient conditions under high light was not largely affected (Figure 4.15). Carbon supplementation yielded more saturates, a common observation in published research (Perez-Garcia *et al.*, 2011). A difference can be seen between WT and PM in standard BBM media, PM containing more $C_{18:3}$ and less $C_{16:0}$. A difference is also seen in WT and PM for BBM C+ conditions with WT capable of accumulating more saturated $C_{18:0}$ than PM, with a reduction in $C_{16:0}$. PM accumulated more $C_{20:0}$ than WT. Full GC-MS data is presented in Appendix B.

4.3 Discussion

The genetic modification of microalgal species is believed to have had a significant impact on in the field of algal biotechnology (Flynn *et al.,* 2010). The cell wall provides a significant energy barrier to the extraction of products from microalgae (Razon and Tan, 2011). This Chapter describes the attempts to generate mutants using EMS and UV and staining for cell wall mutants using staining and FACS. UV was found to be a repeatable method for generating mutants, yet no viable cell wall mutants were yielded. However a pale mutant was generated using UV and subjected to further analysis. The high-throughput mutant screening method using FACS required further development.

4.3.1 Mutagenesis and screening

EMS mutagenesis was highly variable in the effect it had on all strains that underwent mutagenesis with the chemical. Large quantities were needed for *P. ellipsoidea* and was deemed unsafe, with the method requiring a lot of development despite its common use as a mutagen for other organisms. The clustering of exponential phase cells of *P. ellipsoidea* could have accounted for the 'observed' high survival rate as healthy cells may have clustered with dead cells, affecting the reality of the 10% survival calibration. An attempt to remedy this has undergone investigation by Kaloudis (2011); prior sonication may help to break up cells for mutagenesis, however his results still showed a large fluctuation in survival values. Upon closer observation of product details from Sigma, it is suggested to store EMS at room temperature, despite having a half-life of 48hr at 25°C. UV mutagenesis may have yielded fewer mutants than EMS, as algae have excellent UV repair mechanisms due to their evolutionary history (Lambert *et al.,* 1980). However a pale mutant was yielded from UV mutagenesis of *C. emersonii*, which was further characterised.

No viable cell wall mutants were found using FACS, which only underwent a preliminary investigation of method development. This method may have required multiple rounds of FACS before a phenotypically distinct population emerged. The accumulation of cell walls in the media (a common observation with algaenan-containing species (Burczyk *et al.,* 1999) may have led to a high level of false positives. Regrettably due to time constraints, no further development of the FACS assay was carried out.

4.3.2 Pale mutant characterisation

Results of the pale mutant (PM) characterisation suggest that PM is only a photosynthetic mutant, and mutated in its photosynthetic machinery. The difference in colour suggests a reduction in one or more pigments, which may explain its lowered productivity when under high light. In lowered light its growth despite a delay is close to WT. Under lowered light conditions cell counts are similar between PM and WT.

It was initially thought that PM may also be a metabolic mutant, as it appeared to grow better on carbon supplemented agar in darker light than the WT. If this were correct it may have exhibited higher lipid accumulation than WT and/or more saturated FAMEs. Autotrophic cultures form more unsaturated (polar) lipids, whereas heterotrophic conditions form more saturated (non-polar) lipids (Perez-Garcia *et al.,* 2011). Yet product analyses of PM and WT under high light in different media, suggested it is simply pushed to its heterotrophic limits. There are no large differences in lipid profile and lipid content is consistently lower in PM compared to WT. This reduction in lipid content may be due to the increased repair activity or poor health of the PM mutant. It would have been interesting to screen products of WT and PM under a lowered light conditions, yet time and space restrictions meant this was not possible.

4.3.3 Further work

Ideally EMS as a mutagen should be further investigated. UV may have yielded lower numbers of mutants due to inherent and effective UV-specific DNA repair mechanisms present in microalgae. It may be interesting to determine a 'Mutagenic Index', (i.e. a number for unusual looking colonies per 1000 colonies at 10% survival) for different mutagens. The FACS assay should be further developed as it still has potential. Kaloudis (2011) subjected *P. ellipsoidea* 'ni' to further rounds of FACS and despite many rounds still did not yield any cell wall mutants of *P. ellipsoidea*. This may be due to *P. ellipsoidea* having a very thin cell wall potentially consisting of mostly algaenan (Chapter 3.2.5) and may not be the case for algaenan deficient cells of *C. emersonii*. Selecting for cell wall mutants may simply require many rounds of selection. Should any future viable cell wall mutants be isolated they could be screened with sonication, as described in Chapter 3.2.6.

4.3.4 Summary

EMS proved too variable for calibrating for use with our strains. UV was repeatable but may have yielded a low level of mutants due to inherent DNA repair mechanisms in algae. Using FACS as an assay for mutants has much potential, yet requires optimisation to reduce the number of false positives. The characterisation of the PM of *C. emersonii* demonstrated that thorough investigation of a potential 'desired candidate' is required to determine any negative phenotypes, which may influence its advantages over a WT. Further investigation of the FAME content and profile of PM would be desirable to determine the effects of lowered light on the mutant compared to WT.

5. BIOPROSPECTING THE ROMAN BATHS FOR THERMOTOLERANT MICROALGAE

5.1 Introduction

5.1.1 Benefits of 'phycoprospecting'

'Phycoprospecting' is the isolation of algae with intrinsic characteristics favourable to bioresource production and/or waste mitigation, from different aquatic environments (Wilkie *et al.,* 2011). Algae are relatively simple in their requirements compared to traditional crops requiring only light, basic micronutrients, CO_2, and are capable of synthesising a variety of products (Mutanda *et al.,* 2011). As discussed in Chapter 1.2.1, microalgae are incredibly diverse and currently seen as an untapped resource (Pulz and Gross, 2004). However only ~15 species are used commercially, primarily for neutraceuticals and animal feed. As such phycoprospecting is a very useful tool for global algae-based bioresource development (Wilkie *et al.,* 2011).

There are believed to be between 200,000-800,000 species (Ratha and Prassana, 2012), with the most globally abundant groups consisting of diatoms (100,000 species) green algae (8000), blue green (2000) and golden algae (1000) (Mutanda *et al.,* 2011). They are found in diverse environments from freshwater to hyper saline, waste streams, ponds and coastal areas. Current culture collections only contain fraction of estimated species, with many libraries holding duplicates (Parmar *et al.,* 2011). The Aquatic Screening Program during the 1980-1990s (Scott *et al.,* 2010) highlighted the wide variability of algal morphology (existing as single cells, filaments, aggregates, colonies, branched chains, microbial mats), biology and ecology during its search for the ideal 'biofuel alga'. During this time Japan was also heavily researching algae (Ratha and Prasanna, 2012), hence the majority of publications from this era were from Japan and the US. Interest in microalgae for biotechnology has only recently resurged due to peak oil and limited resources, which is reflected in the infancy of algal research and culture technologies (Pienkos and Darzins, 2009).

If there is an end application in mind, it is beneficial to bioprospect regions which may already mimic conditions favouring the growth of algae with a desired characteristic. For example an alga is desired which can bioaccumulate a heavy metal, it is worth searching areas known to naturally be higher in that metal. Or if a culture system is to be set up along the coast, it would make financial sense to find a strain that could utilise seawater as a culture

medium (Ali, 2009). Known oleaginaceous algae vary greatly in their growth rates, FAME profile and lipid content, and tend to accumulate lipid in stationary phase often as a result of stress, which can be induced by altering culture conditions (Ali, 2009). Likely places to find oleaginaceous algae are 'boom and bust' environments, where algae must fix and store large quantities of carbon in the form of lipid reserves until the next nutrient burst (Ali, 2009).

Fatty acids are vital in determining quality of biodiesel produced. Therefore, it would be beneficial to rapidly screen algae from environmental samples for their lipid content and FAME profile (Figure 5.1). Accurate lipid profiling of algae is usually carried out using GC-MS, which not only requires a larger sample of biomass but is also coupled with lengthy sample preparation often involving lyophilisation, extraction, purification and transesterification (Mutanda *et al.,* 2011). Lipid extraction methods have been evaluated (described in Chapter 7) in an attempt to shorten and simplify the extraction of FAMEs from algal samples in order to more rapidly obtain experimental results. Nile red has been successfully used in conjunction with a fluorescence activated cell sorter (FACS) to act as a quick screen to isolate lipid-rich cells. Fluorescence has been shown to strongly correlate with lipid content, yet requires prior construction of species specific calibration curves (Mutanda *et al.,* 2011). Plymouth Marine Laboratories (PML) are currently undertaking a global microalgal bioprospecting project. Their preferred method is to collect small water samples from surface waters (~30m deep, the maximum for photosynthesis), culturing in low light with added nutrients and regular subculturing (allowing for the selection of fast and slow growing species). FACS has been implemented at PML to isolate cells based on fluorescent properties of chlorophyll and fluorescent stains into 96 well plates (the fluorescent stain signal in the FACS is not related to lipid content) (Ali, 2009). However nile red has an emission frequency range which overlaps with chlorophyll fluorescence in healthy photosynthesising cells. BODIPY (a relatively recent fluorescent lipid stain) is being used in preference to nile red for the isolation of oleaginaceous species as it has a narrower emission frequency range than nile red (Ratha and Prasanna, 2012).

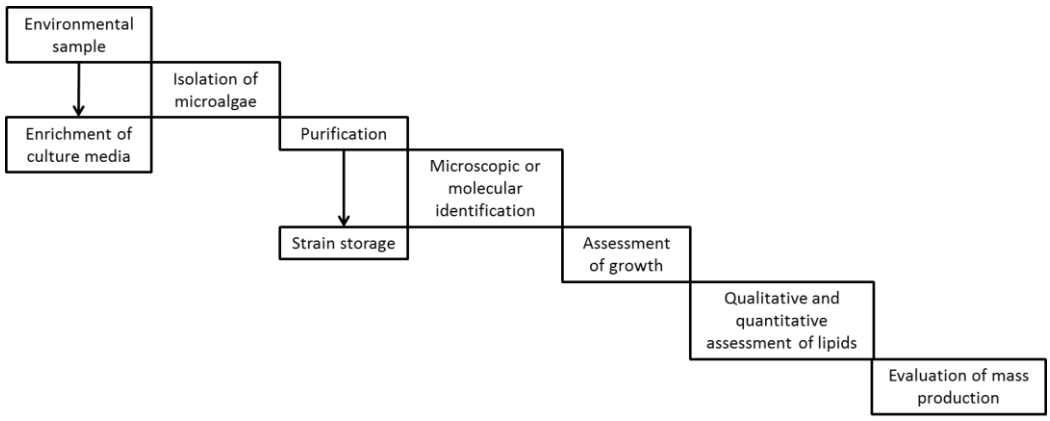


Figure 5.1: Schematic of a phycoprospecting procedure for the isolation of biodiesel-producing microalgae.
Adapted from Mutanda *et al.* (2011).

When cultured locally, native algae should be adapted to existing biotic and abiotic factors and may help prevent invasion of other algae if grown in ponds. For example algae native to natural waste streams, should be suitable for growth in waste water and may be better at cleaning water/bioremediation than other strains (Wilkie *et al.,* 2011). With this in mind it is important to find a strain which is still capable of being very productive (high doublings in biomass and high content of desired products). There could potentially be a trade-off in how productive the strain is versus culturing benefits (e.g. if a culture remains 'cleaner' yet less productive at a higher pH), which may reduce overall cost and still make the overall process lucrative (Ali, 2009).

Harvesting costs should be taken into account when looking for new strains. Those which are filamentous, large celled, autoflocculate or with high 'specific gravity' compared to the mediums could potentially lower harvesting costs (Mutanda *et al.,* 2011). For example filamentous algae such as *Oscillatoria* spp., are desirable for large scale cultivation due to the ease of dewatering using low energy and low cost methods such as filtration (Mohan *et al.,* 2010), this would be beneficial to most current culture systems (Pulz and Gross, 2004).

Selective pressures and changing environmental conditions mean there are a range of microalgae found in extreme environments worldwide (Mutanda *et al.,* 2011). Traditional regions to investigate for novel algae to be used commercially are hypersaline environments and thermophilic springs. These robust habitats may offer algae better adapted to specific conditions and may increase the chances of isolating unique hyper-lipid producing microalgae. This is due to the protection such an 'extreme' environment offers from

contamination and domination of a culture by invasive local algae or contaminants (Ali, 2009). This is most important in the use of open ponds (cheapest culture method) but could also apply to closed systems where wastewater is used (usually requiring prior sterilisation) (Wilkie *et al.*, 2011).

Temperature tolerant algae are desirable for reducing culture cooling costs, which can reach algal-lethal high temperatures in both open and closed systems using natural sunlight or flue gas as a feed (Chapter 1.3.2). In addition, there are strong applications for thermostable enzymes (e.g. Taq polymerase for PCR). An overwhelming number of global biorefineries require the use of thermostable enzymes which remain active and/or stable at high temperatures (high mass transfer rates, better substrate solubility and easy mixing). They are also more environmentally friendly than chemical catalysts which often contain expensive toxic compounds. A wide range of reactions can be catalysed using enzymes, some of which have been cloned and successfully expressed in mesophillic organisms such as *Eschericha coli* and *Saccharomyces cervisiae.* Thermophilic organisms are therefore seen as a source of novel enzymes for biocatalysis (e.g. for lignocellulose and starch conversions) (Turner *et al.*, 2007).

5.1.2 Temperature tolerance and the Roman Baths of Aquae Sulis

Temperature control in algal systems can add to cost as algae typically grow optimally at a low temperature range. Open ponds and closed systems can be prone to temperature spikes, which have the potential to wipe out entire cultures (Huang *et al.*, 2012). An ability to tolerate if not thrive in a warm environment would therefore be beneficial. The most commonly cultured microalgae grow best between 16-27ºC (varies with species, strain and culture conditions). Temperatures of <16ºC often slow growth, whereas temperatures of >35ºC are lethal for many species. However, heterotrophic eubacteria can grow up to 95ºC and archea to 114ºC (Barsanti and Gualtieri, 2006). Due to the clarity of hot springs, algae derived from hot springs may have adaptions to reduce the effect of photoinhibition (Castenholz, 1969) (Chapter 1.2.3). Such adaptations may include improved DNA repair damage systems or a reduction in photosynthetic pigments.

There is a general consensus that bacteria evolved in warm environments and many are capable of functioning well at high temperature. There is strong evidence to suggest that more thermotolerant lineages evolved from less thermotolerant ancestors (Miller and Castenholz,

2000). The most thermophilic cyanobacterium can only survive up to 74ºC (*Thermosynechococcus* spp., abundant in Yellowstone, USA and Iceland) and photosynthetic eukaryotes are even less temperature tolerant with the most thermophilic only able to survive up to 57ºC (*C.caldarium* a unicellular rhodophyta), same range as protozoa and fungi (Seckbach, 2007). Filamentous cyanobacteria appear less thermotolerant (*Mastigocladus laminosus* has an upper limit of 58ºC and thermophilic *Oscillatoria* spp. 55-62ºC). Yet some thermotolerant filamentous species are capable of fixing nitrogen (e.g. *M. laminosus*) via specialised structures known as heterocysts. This is an additional advantage when looking for novel strains as it could significantly reduce the environmental and financial cost of fertilizers (Chapter 1.2.3). In addition, thermophilic cyanobacteria can synthesise other valuable products and may be advantageous over green algae for the development of biofuel, possessing fast growth rates under low light, capable of high culture densities and may also be easier to genetically transform (Chapter 3.1). Thermophilic microorganisms are also capable of utilizing a variety of complex carbohydrates and their enzymes are of great value to industry (Blumer-Schuette et. al., 2008).

Eukaryotic algae appear absent from environments with temperatures above 60ºC, this is believed to be due to an inability to form thermostable functional organellar membranes (Tansey and Brock, 1972). *Chlorella* sp. was studied after heat shock treatment and shown to undergo programmed cell death (DNA fragmentation, cell shrinkage, detachment of plasma membrane from cell wall, with alteration to chloroplast morphology) (Zuppini *et al.*, 2007), with the first noticeable degradation being of photosynthetic pigments and DNA. Unlike plants however, *Chlorella* sp. cells maintained cell membrane integrity (Leu and Hu, 2005).

Microbial mats are important primary producers in extreme habitats they also play an important role in stabilization of micro-environments deeming them more suitable to microbial growth (Pattanaik *et al.*, 2008). As a result algae from warm environments tend to exist in close-knit diverse communities, aiding stabilisation by establishing oxygen gradients and supplying nutrients to bacteria (particularly in the case of nitrogen fixing cyanobacteria in nutrient deficient environments). Organic matter produced by these mats will additionally 'feed' other organisms, resulting in microbial mat communities capable of establishing themselves in a wider range of environments than the individual species alone (Abed and Köster, 2005). In essence, it is important to note that the population dynamics of microalgae in their natural environments is complex (i.e. lower winter temperatures and light may

encourage benthic strains such as *Scenedesmus* sp. whereas summer may enhance chlorophyte growth such as *Chlorella* sp. (Mutanda *et al.,* 2011). An algal community is a 'hysteretic system' with two alternative equilibria. Eukaryotic green algae tend to dominate under high light conditions whereas cyanobacteria outcompete green algae under low light conditions (Scheffer *et al.,* 1997). Cyanobacteria are often found in warmer and lower nutrient environments that eukaryotic algae (de Winder *et al.,* 1990). With this in mind cyanobacteria are likely to outnumber eukaryotic algae in the Roman Baths.

5.1.3 The Roman Baths of Aquae Sulis

The largest geothermal spring in the UK is situated in the city of Bath at the Roman Baths (ST750647) (Atkinson and Davison, 2002). The hot springs of Bath comprise three springs; the Kings spring, Hetling spring and Cross Bath spring (recently reopened as a tourist attraction) (Kellaway, 1991). The baths themselves were gradually constructed by roman settlers between 70-370AD, falling into disrepair and gradually became buried during the 5th century as the Romans withdrew from Britain (Byrne, 2008). Excavation in 1878 after accidental discovery during building work, reopened the complex (Kellaway, 1991).

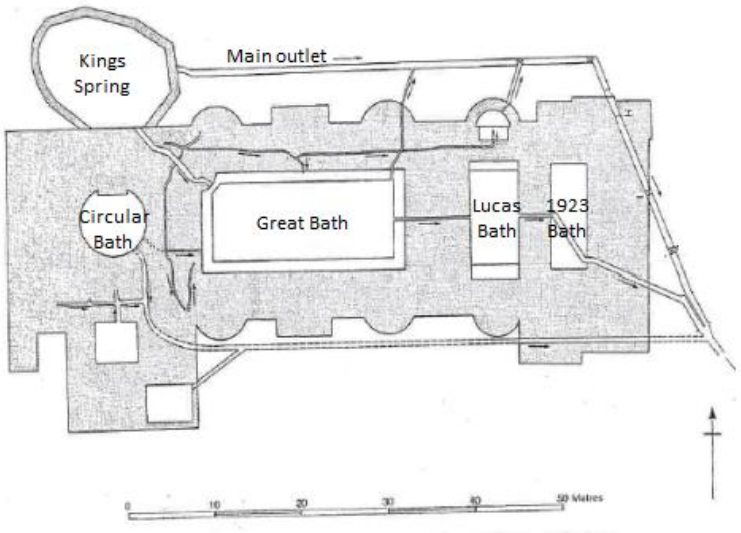


Figure 5.2: Diagram of the Roman Bath waterways showing the Great Bath and Kings Spring (Kings Bath) both of which underwent bioprospecting for microalgae
From Byrne (2008).

The hot waters at Bath are believed to have originated from rain falling in the Mendip hills (~20km from Bath), travelling though caves in carboniferous limestone underneath Radstock coalfield (at a depth of 2.5km), where under pressure it has acquired heat and risen up though a fault (Kellaway, 1991), before emerging from the Kings Spring beneath the Kings Bath at a temperature of 46.5°C, which records show have been constant since records started in 1754. The hot water erupts from the depths of the Kings Bath (KB, 46.5°C) and drains into the Great Bath (GB, 39.0°C), which can be observed as currents and exholved gas bubbles gently stirring the surface waters (Figure 5.2, Andrews *et al*, 1982). The estimated output of water is 13 l s^{-1} (Andrews *et al.*, 1982) and the residence time of water is ~4,000 yr (Kellaway, 1991).

All the springs are shown to have calcium, sulphate, sodium and chloride at high concentrations and precipitates of iron hydroxides, which cause staining and encrustation of some of the waterways in the baths (Kellaway, 1991). There are also 'metalliferous mineral deposits' which form dependent on water agitation, cooling and oxygenation (Kellaway, 1991). The boreholes were all shown to be microbiologically sterile (Kellaway, 1991), yet the waters of the baths appear to be full of microbial growth. As hot water springs emerge near boiling temperature, flowing water will form a temperature gradient whereby microalgae aggregate according to their preference (Seckbach, 2007). As thermotolerance is an interesting trait to investigate (with added benefits to production of algal biofuel), we set out to bioprospect the hot waters of the Roman Bath for microalgal strains with advantages to produce lipids for biodiesel production.

It is important to note that when bioprospecting it is important to obtain permission of the landowner. In the case of marine bioprospecting, avoiding 'biopiracy' becomes a complicated affair (Ali, 2011). In the search of thermotolerant microalgae we have had exclusive permission from the Roman Baths (Tom Byrne), to prospect the thermal waters for this project.

5.1.4 The challenges of isolation

Common methods for isolation and identification all come with benefits and disadvantages dependent on the species to be isolated and the technique involved (Table 5.1).

Table 5.1: A summary of advantages and disadvantages of different microalgal isolation techniques. Adapted from Mutanda *et al.* (2011).

Technique	Advantages	Disadvantages
Micropipette	Single cells can be transferred and purified	Laborious, time consuming, need skills difficult for small/flagellated, cell damage
Agar plating	Easy	Not for flagellates or sensitive algae, outcompete
Serial dilution	Easy	Unsuitable when algae are outweighed by bacteria
Differential centrifugation	Less damaging to sensitive cells	Expensive
Filtration	Better separation of bacteria	Not suitable for small algae or those secreting mucilage (embedded bacteria)
Antibiotics	Relatively easy, low cost	Damaging to photosynthetic machinery and can lead to resistance in contaminants. Time consuming due to necessary calibration.
Flow cytometry	Precise and rapid, good for small delicate taxa, get cell info, axenic, use directly on natural samples	Expensive equipment and operation, often centralised and 'multi user'
Ultrasonication	Good for separating bacteria	Can damage cells and not 'standalone method'

Antibiotics can be added to discourage growth of bacteria or contaminating cyanobacteria but typically cause extensive chloroplast damage (Vázquez-Martínez *et al.,* 2004). The use of antibiotics although efficient at eliminating contaminants, requires meticulous calibration to determine the minimal lethal concentration of the isolate, which will vary for different antibiotics between strains (Choi *et al.,* 2008). In some cases the addition of antibiotics may promote the growth of an unwanted microorganism (e.g. ampicillin and penicillin G in an algal isolation experiment conducted by Ferris and Hirsch (1991)).

Media enrichments can be detrimental to the isolation of microalgae if not introduced gradually, as some algae require an 'adjustment period' depend on the death of other organisms as a substrate for growth (Mutanda *et al.,* 2011). Chemical agents harmless to microalgae but harmful to contaminants, can also be employed (e.g. phenol, sodium hypochlorite, detergents, sodium sulphide, elevated temperatures, high or low pH, UV or gamma radiation) (Ferris and Hirsch, 1991).

Gravimetric methods such as centrifugation with a density gradient media (i.e. Percoll) can aid the isolation process but cannot be used alone in reducing the level of contamination if it is high. Micropipetting is a simple isolation method for physically robust algae that can survive shear stress. However great skill and patience is required and this method is only

applicable to single celled algae. Flow cytometry can be used to isolate algal cells based on autofluorescence, yet this is less successful for cultures with heavy bacterial contamination and again can only be applied to single cells (Mutanda *et al.*, 2011).

For filamentous species, bacterial cells are very strongly associated with the secreted mucilage that coats algal filaments, which makes the process of obtaining axenic cultures very difficult. Fragmentation by sonication and repeated washing of cyanobacterial filament fragments had limited success in reducing the contaminants from field samples. This method was shown to be too crude for the removal of all contaminating cells from a filament fragment (Vázquez-Martínez *et al.*, 2004).

Dilution and agar plating is an old method commonly used for coccoid algae. Some do not grow on the surface but only when embedded in agar. Agar is a natural product derived from algae and as such contains metabolites or hormones which may inhibit growth of some microalgae (Issa, 1999) and encourage growth of contaminating bacteria (by containing nutrient traces). This can result in dominance of plates by fungi or bacteria before algal cells have had the opportunity to grow (Ferris and Hirsch, 1991). A recent study by Pereira *et al.* (2012) used the lipid probe BODIPY and FACS to isolate lipid-rich marine microalgae, and found that microplating directly onto agar accelerated algal growth whereas liquid cultures were found to have higher levels of bacteria and lower levels of algae (Pereira *et al.*, 2012).

A unique ‘plate scoring method’ to isolate filamentous cyanobacteria was devised by Vaara *et al* (1979). ‘Scored’ agar plates and the application of unidirectional light led to rapid separation of filamentous cyanobacteria from contaminant bacteria. This worked by the gliding motion of cyanobacteria though scores made in the agar (shedding bacterial contaminants stuck to the outer mucilage) towards a light source. Clean filaments could then be picked off for further culturing. However it was found that some strains of *Oscillatoria* spp. and *Anabaena* spp. cannot grow even in ideal medium (due to absence of ‘contaminating’ bacteria). In other studies bacteria are often found to be closely associated with the muciferous layer of the algal cell walls (Croft *et al.*, 2005). This is a common problem when isolating cyanobacteria as they have a tendency to form complex relationships with other organisms (Ferris and Hirsch, 1991).

Another novel method for isolating algae from environmental samples (the 'filter paper method') was described by Ferris and Hirsch (1991). By using stacks of sterilised glass fibre filter paper inside Petri dishes, they were able to create an 'imitation agar plate' by saturating it with the relevant media. This circumvents the problem of impure agar, more closely mimicking a 'clean' wet rocky surface (with no carbon source available this is advantageous for c-fixing algae than bacterial or fungal contaminants). The method described resulted in up to a 15-fold reduction in the growth of accompanying bacteria. In addition, twice the number of colony forming units per sample were formed on glass fibre medium compared to agar (Ferris and Hirsch, 1991).

It is advisable to mimic the environmental conditions from which the organisms were isolated. It is best to gradually alter growth media to improve chances of isolation and therefore it is critical to measure parameters on site along GPS coordinates (i.e. light (quality and quantity) water temperature, nutrient concentrations (nitrates and phosphates), dissolved O_2, dissolved CO_2, pH salinity and where possible water velocity (flowing water) and depth). It is also important to note other features of interest such as competitors (bacteria, fungi, and zooplankton) (Mutanda *et al.,* 2011).

5.1.5 Identifying isolates

Identification of most biological samples often involves a combination of morphological examination and genetic characterization (Mutanda *et al.,* 2011) as many species lack distinct morphological features, making them hard to identify (Pulz and Gross, 2004). Algal cells can also change shape and size during their lifecycle and under certain growth conditions, as such morphology can be misleading (applicable to many coccoid algae (Surek, 2008)).

Dyes are argued to be a more sensitive reagent for the study of cells and organelles of microorganisms. Staining with dyes may help distinguish some algal groups, yet many species (e.g. *Chlorella* spp., *Scenedesmus* spp., *Haematococcus* spp.) are impenetrable to certain dyes usually due to the morphology of the cell wall (Chapter 3). As a result the screening of oleaginaceous species using nile red-staining may prove ineffective (Gao *et al*, 2008). Many algae are affected by preparation or fixation so often cannot be identified in this way (Mutanda *et al.*, 2011).

Molecular techniques are relatively rapid and commonly used as a means of identification. Common regions used are: rRNA genes, mitochondrial genes, plastid genes (rbcL), ITS, and microsatellite DNA sequences. Smaller rDNA sequences (16S/18S) are good for identifying distantly related species, whereas larger rDNA sequences (23S/28S) are useful for distinguishing closely related species (Mutanda *et al.,* 2011). The mitochondrial (cox1) marker had gained recognition as the 'standard' DNA barcode marker for many species. However, it is not suitable for green algae and higher plants (as the rate of evolution in these groups is too slow). The other alternatives have remained unpopular, yet 18S and 16S rDNA genes are most widely used for the identification of algae and cyanobacteria to date (Satoh *et al.,* 2011).

The problem of identification based on one gene is that unicellular algae tend to have low 18S divergences, suggesting that small rDNA genes are too conservative to assess planktonic eukaryotic diversity. It is anticipated that the biodiversity of single-celled eukaryotic species is underestimated, with numerous 'cryptic species' (Piganeau *et al.,* 2011). For example, 16S rDNA gene sequences from 7 geographically distant strains of *M. chthonoplastes* were compared and found to be indistinguishable from another despite very coherent sequences (Garcia-Pichel *et al.,* 1996). Ideally robust libraries of genetic data would be available with which to compare isolates. Unfortunately there are still very few barcodes available due to the nature of algal DNA and the infancy of its biotechnology (Surek, 2008). The goal of the International Barcoding Initiative (IBI) is to find a single universal short DNA fragment which gives clear identification of species (Surek, 2008).

There are still many questions facing the methods used to identify algae. Should we use genetic 'barcodes'? 'Should we omit morphology? Are we generating superfluous data without taxonomy? How do we progress in interpreting genetic differences? Is a single marker adequate for algae? At present a 'polyphasic' approach using molecular and morphological data is found to be more robust and is preferred (Surek, 2008).

Conditions in the Baths are unique in the UK. This may have proved an opportunity for establishment of unusual strains, which may possess uncommon features. Due to the difficult nature of identifying algae based on morphology alone, microscopy may be useful in identifying between prokaryotic and eukaryotic cells, yet species level identification is best undertaken using molecular methods. 16S and 18S rDNA sequencing is implemented at PML

to confirm identity of isolates or to compare to uncultivated strains (Ali, 2011). As 16S and 18S rDNA sequences are commonly used this is the method employed in this project.

5.2 Results

5.2.1 Observations Aquae Sulis

In order to aid microalgal isolation efforts and/or account for any observed behaviour of isolated algae, the aquatic environment of the Roman Baths underwent visual and chemical analysis. The waters of the Roman Baths upon first glance appear a striking milky blue-green colour, largely attributed to the suspension of fine particles in the water and reflection and absorption of natural sunlight (resulting in a shallow depth of visibility of ~30cm). Observations found extensive microbial mat communities submerged beneath the water surface, some with striking blue-green patches and distinctive filamentous 'hairy' brown mats which grew around evolved gas bubbles. These eventually create gelatinous 'balloons' which rise to the surface, anchored to the microbial mat below by filamentous 'ropes'. Comprising a significantly thick layer between the mats and rocky surfaces is a bright orange mud-like residue, likely caused by bacteria, detritus and iron hydroxide precipitates (Kellaway, 1991), which can be seen staining some of the waterways in the baths (Figure 5.3).

Figure 5.3: A typical Roman Bath microbial mat community.
The orange sub-layer comprises of compact masses of rod-shaped heterotrophic carotenoid rich bacteria and detritus, supporting the growth of filamentous algae on the upper surface of the mat. Trapped bubbles of evolved gas can also be seen.

In addition to iron, the springs also have high concentrations of calcium, sulphate, sodium and chloride (Kellaway, 1991). Excess iron has been shown to have a negative effect on phytoplankton growth (decreased ribosomes, more lipid radicals, alterations in starch deposits) increasing oxidative stress (Estevez *et al.,* 2001). There are also visible mineral

deposits, which form dependent on water agitation, cooling and oxygenation (Kellaway, 1991). Although records showed that chemical composition of the Bath waters had remained stable since records began in 1874, for the purposes of this project water samples from the Kings Bath (KB) and the Great Bath (GB) were sent for chemical analysis. Water analyses of the Great Bath (GB) and Kings Bath (KB) showed that abiotic conditions in the Baths have remained stable (Table 5.2). The temperature of the Great Bath was found to be 39.0°C and the Kings Bath 45.0 °C.

Table 5.2: Chemical composition, pH and temperatures of the Great Bath (GB) and Kings Bath (KB)) compared to historical measurements from the Kings spring. Analysis performed by Severn Trent Services (unless stated), all data in mg l^{-1}, other data from Kellaway, (1991).

Elements	**1874**	**1888**	**1936**	**1961**	**1979**	**1986**	**2011 GB**	**2011 KB**
B					0.59		0.47	0.51
Ca	377	402	392	392	382	390	394	421
Cu					0.002		0.002	0.003
Fe					0.88		0.25	0.39
Mg	47	52	51	54	53	58	51	53
Mn					0.068		0.135	0.140
Mo					<0.1		<0.002	<0.002
K	39	31	15.4	15.7	17.4	18.1	20.1	20.8
Na	129	135	177	174	183	228	196	203
Cl	280	277	274	276	287	335	332	330
NO_3^-N					<0.1			
HCO_3	86	88	193	216	192	187		
SiO_2					20.6 (Si)		42.99	42.27
SO_4	869	1061	1001	1021	1032	1030	978	977
BOD							<1	<1
pH					6.65			
Temp °C	46	47	49	48	45.3	43.5	39.0	45.0

5.2.2 Isolation methods (unialgal)

To determine the diversity of microalgae present in the Roman Baths, multiple scrapings and water samples were taken at different depths and locations in the Baths. Initial scrapings and water samples were taken and analysed using simple light microscopy at various magnifications to ascertain whether many algal species were present (and in what quantities), before attempting isolation. These samples were taken from the Great Bath (39^{o}C), Kings Bath (46^{o}C), Sacred Spring (25^{o}C) and an underground location with very low light (<20^{o}C) Many morphologically diverse species were found; two dominant filamentous species and many single celled species. Using light microscopy alone, it became apparent that the majority of species found were cyanobacterial (Figures 5.4 - 5.7).

Figure 5.4: Light microscope images of algae found during preliminary sampling of the Great Bath (39ºC).
Top left image is a species found to dominate the microbial mat community (isolated and identified as *Oscillatoria sancta*). Bar = 20µm.

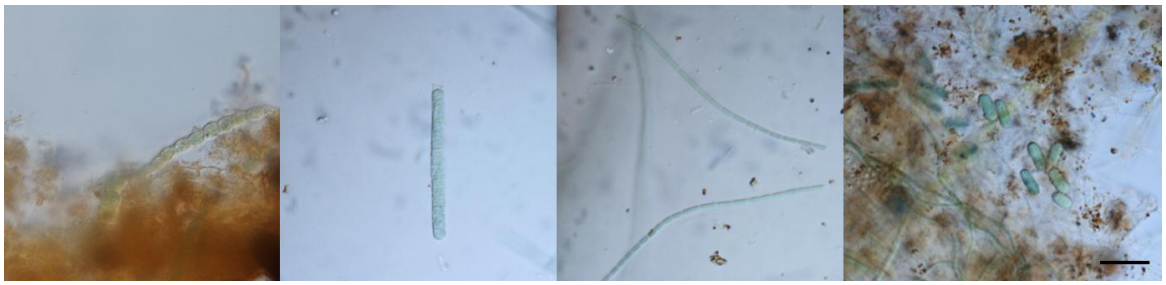


Figure 5.6: Light microscope images of algae found during preliminary sampling of the Kings Bath (46ºC).
Image second from right is a species found to dominate microbial mat communities (isolated and identified as *Microcoleus chthonoplastes*). Bar = 20µm.

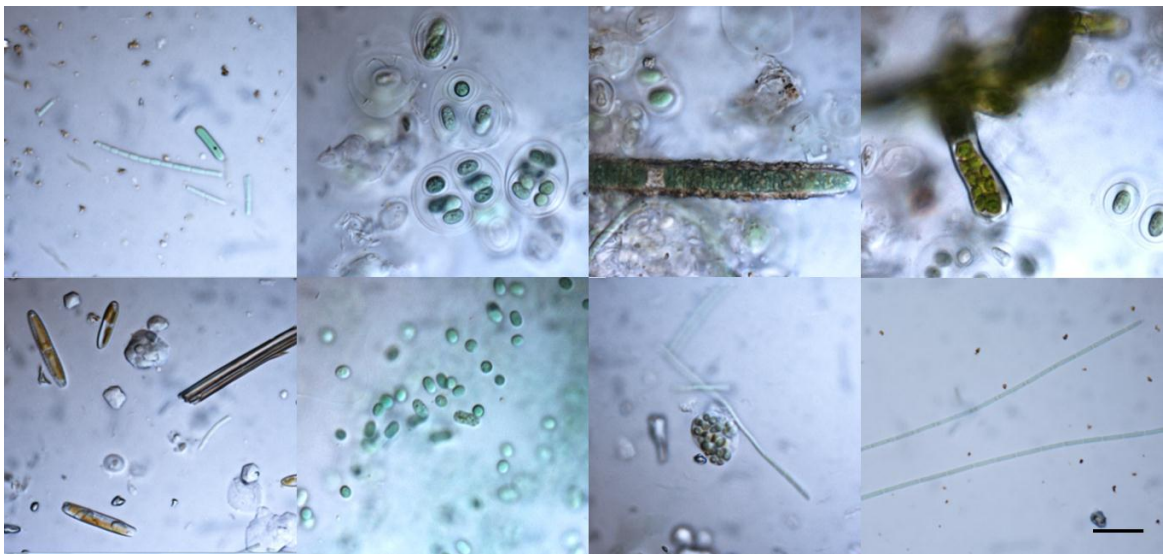


Figure 5.7: Light microscope images of algae found during preliminary sampling of the Sacred Spring (25ºC).
Bar = 20µm.

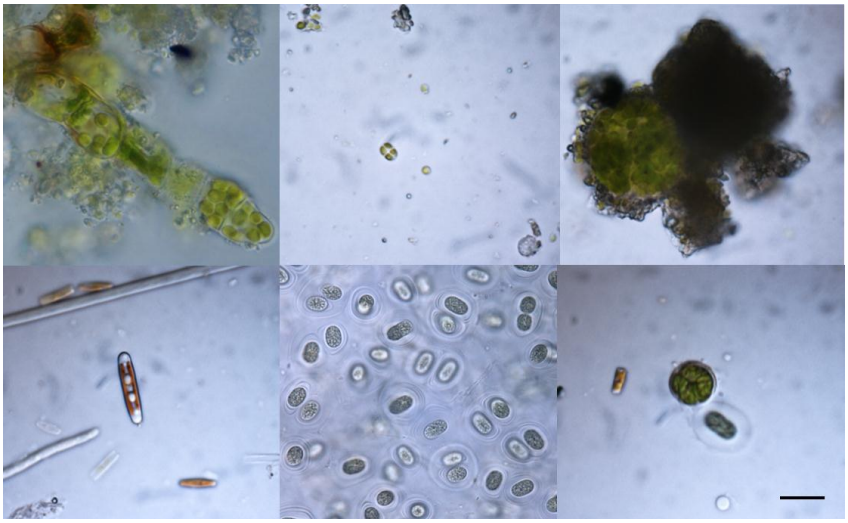


Figure 5.8: Light microscope images of algae found during preliminary sampling of an underground location beneath Stall Street, Bath (<20ºC).
Bar = 20µm.

It was decided to focus efforts on isolating microalgae from the two warmest locations (the Kings Baths (46^{o}C) and Great Bath (39^{o}C)) in the interest of finding the most thermotolerant algae. These locations were also found to contain a higher diversity of microorganisms. From these early investigations, the dominant microalgae found in the Great Bath (later identified as *Oscillatoria sancta*, Figure 5.9) and Kings Bath (later identified as *Microcoleus chthonoplastes*, Figure 5.9) were used to determine suitable methods for isolating and cleaning samples (Chapter 5.2.3). Another dominant filamentous microalga (sampled by Dr. Heather MacDonald, University of the West of England) was also donated to the project at Bath (Figure 5.9). This heavily contaminated isolate was subjected to preliminary isolation experiments alongside *O. sancta* and *M. chthonoplastes.*

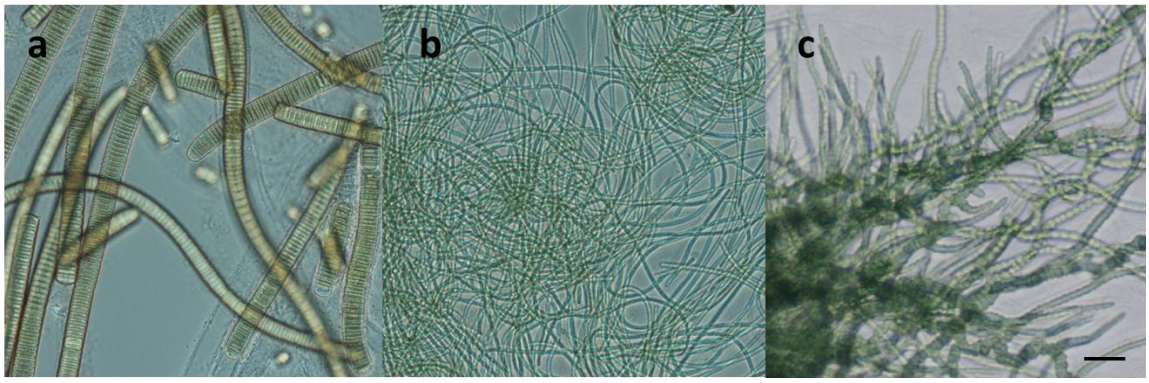


Figure 5.9: Light microscope images of dominant filamentous species found in Roman Bath microbial mats.
These were later identified as (a) *Oscillatoria sancta*, (b) *Microcoleus chthonoplastes* and (c) *Mastigocladus laminosus*) isolated from scrapings. Bar =20µm.

Micropipetting was initially tested as a means of crudely isolating single cells, yet was deemed unsuccessful and highly labour intensive. This may have been a result of progressive shear damage caused to cells with each transfer into a clean well. Isolating short sections of filamentous algae may have also not been sufficient for regrowth.

Samples observed by light microscopy showed that other single celled microorganisms (algae or zooplankton) exist in very low densities in the waters when not part of a microbial mat. Therefore an experiment was devised to filter large quantities of water in order to capture any single-celled algae. Approximately 30l of water from the Great Bath was filtered through a custom made filter-unit (comprising three 'grades', described in Chapter 2.2.17), with any fine suspended particles and cells trapped onto Whatman GF filter paper. In order to clean all isolates from contaminant fungal spores and bacteria, samples underwent serial culturing on Whatman GF filter paper stacks ('filter paper method', described in Chapter 2.2.19) saturated in medium. This method yielded colonies of species *Coelastrella saipanensis*, *Klebsormidium* sp., *Hantzschia* sp. and *Chroococcidiopsis thermalis* (shown in Figure 5.10).

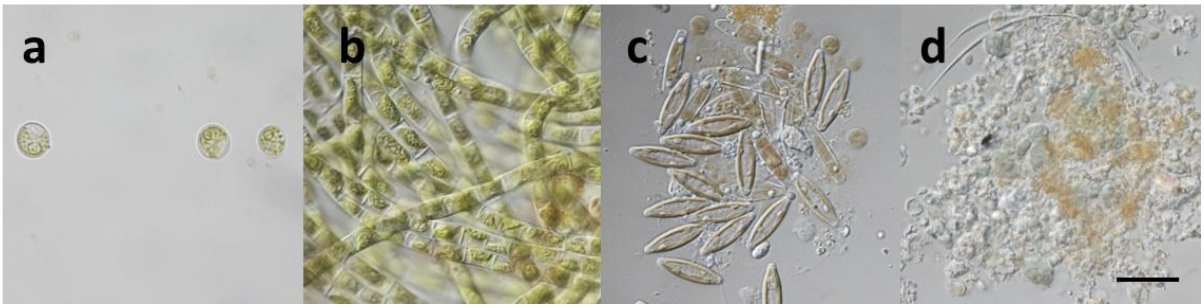


Figure 5.10: Light microscope images of (a) *Coelastrella saipanensis*, (b) *Klebsormidium* sp., (c) *Hantzschia* sp. and (d) *Chroococcidiopsis thermalis*.
These were isolated as colonies, picked off filter paper using the 'filter stack method'. Bar =20µm.

5.2.3 Decontamination of uni-algal cultures from Roman Bath samples

In order to identify Roman Bath isolates by 16S (for cyanobacteria) or 18S rDNA (for eukaryotic algae) 'barcoding', it was necessary to remove any prokaryotic and eukaryotic contaminants. This was visually assessed under the light microscope.

Dominant filamentous species (shown in Figure 5.9) were used to examine various 'clean-up' isolation methods, due to their availability in the Baths. Firstly, their media preference was determined. BBM, D medium (suggested by Castenholz, 1969) and autoclaved Roman Bath borehole water, were tested in agar plate form and in 100ml liquid media (in 250ml conical

flasks). Samples showed growth on agar plates (made from BBM and autoclaved bath water) but not liquid media. However, growth on agar plates showed a high level of contamination. This was further reduced in *Os*, *Mc* and *Ml* by the use of the 'plate scoring method' (Figure 5.11).

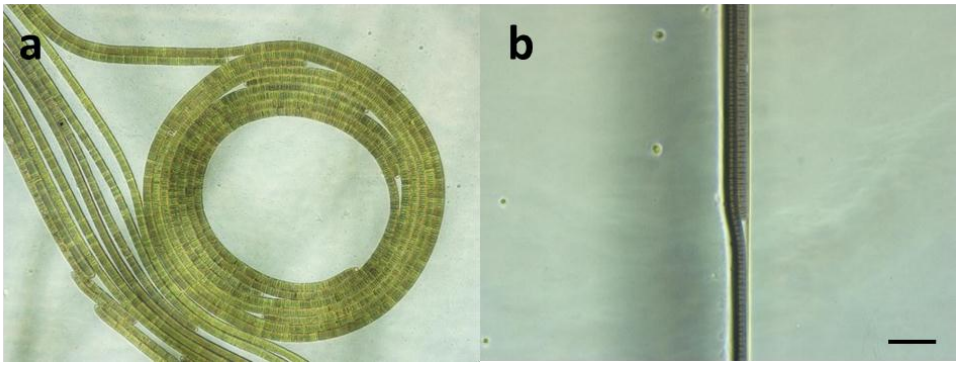


Figure 5.11: Filamentous *O. sancta* (a) showing spiral growth on unscored agar and (b) undergoing decontamination using the 'plate scoring method'.
Agar plates are scored with a sterile razorblade and the algal sample inoculated at one end perpendicular to the direction of scoring. Plates are blacked out with the opposite end to inoculation open to a light source. This encourages filaments to grow toward the light source through the agar scores, sloughing contaminants as they grow. Bar = 40µm.

Despite greatly reducing the level of contamination, some bacteria still remained stuck to the surface of filaments. *Os*, *Mc* and *Ml* also appear to grow very slowly on agar plates likely due to trace contaminants. Both strains showed better growth and lower contamination when cultivated using the 'filter stack method' (Table 5.3).

Table 5.3: Qualitative comparison of microalgal growth of *O. sancta* and *M. chthonoplastes* using the 'filter stack method' and growth on agar plates.
Conditions = 25ºC, 150xg, 200µM photons m^{-2} s^{-1}.

Media	*Os* phyta-agar	*Mc* phyta agar	*Os* filterstack	*Mc* filterstack
Borehole water (autoclaved)	+ (bacteria)	(bacteria)	-	-
BBM pH 6.5	(bacteria)	(bacteria)	++	++

Lack of growth on filter paper saturated with autoclaved borehole water, suggests other organisms are necessary for growth of these filamentous species in the Roman Baths (i.e. a microbial mat community) (Figure 5.12).

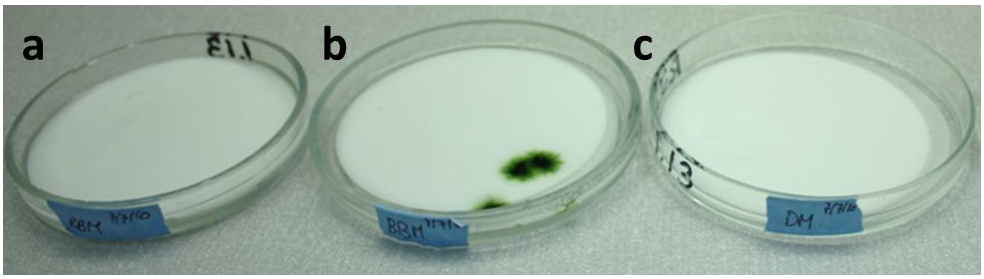


Figure 5.12: *M. chthonoplastes* cultivated on media saturated 'filter stacks', using (a) borehole water, (b) BBM and (c) D media (described by Castenholz, 1969).

Visual appearance of *M. laminosus* changed drastically as it was cleaned of contaminants (particularly a fungus), from green-brown flakes to deep turquoise spheres (Figure 5.13).

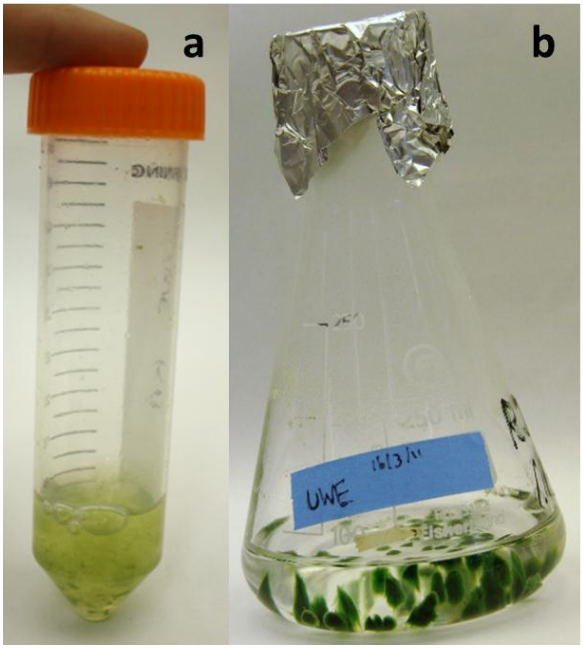


Figure 5.13: Images of *M. laminosus* before (a) and after (b) decontamination.
Prior to decontamination, *M. laminosus* grew with a fungus which altered its morphology (flaky) and colour (brown-green). After decontamination the cyanobacterium grew as deep turquoise spheres.

Once decontaminated, further experiments were carried out on the dominant filamentous isolates, to determine more favourable growth conditions for cyanobacteria, (which typically prefer a higher pH). Increasing the pH of BBM did improve the growth of the filamentous isolates (Table 5.4). BG-11 medium (designed for cyanobacterial culture) proved most successful for growth of these isolates (Table 5.4). As a result, it was decided that eukaryotes were to be cultivated with BBM and cyanobacteria with BG-11.

Table 5.4: Qualitative assessment of growth of *M. chthonoplastes, M. laminosus* and *O. sancta* in different media and at different pH.
Conditions = 28ºC, 150xg, ~100µM photons m^{-2} s^{-1}.

Media	*M. chthonoplastes*	*M. laminosus*	*O. sancta*
BBM pH 5	-	-	-
BBM pH 6.5	-	+	+
BBM pH 8	+	++	+
BG-11 pH8.1	+++	+++	+++

-no growth, + poor growth, ++ good growth, +++vigorous/healthy growth

Klebsormidium sp. (a filamentous eukaryotic alga, Figure 5.10) was decontaminated using the same methods as described for the above filamentous cyanobacteria (with the exception of substituting BG-11 for BBM). Single celled isolates *Coelastrella saipanensis, Hantzschia* sp. and *Chroococcidiopsis thermalis* (Figure 5.10) were decontaminated by serial use of the 'filter stack method' (using BBM, Diatom Media and BG-11 respectively). Final 'proof of purity' of isolates was determined by extensive light microscopy and growth on agar (where applicable).

5.2.4 DNA extraction and clean-up

The procedure of microalgal DNA extraction and PCR amplification of 18S rDNA underwent method development using our stock cultures of *C. vulgaris, C. emersonii* and *S. vacuolatus* (originally donated by UWE as an unidentified green alga 'VT-1'), prior to use on our isolates. Promega GoTaq Green Mastermix was used initially (largely unsuccessfully) and a switch was made to Fermentas DreamTaq Green Mastermix which showed a marked improvement in DNA amplification (brighter PCR product bands).

All algal DNA loaded onto gels was organised into groups with the order of species as follows: *Cv* (*Chlorella vulgaris*), *Ce* (*Chlorella emersonii*), *Sv* (*Scenedesmus vacuolatus* 'VT-1'). DNA quality and quantity varied between different extraction methods used (Figure 5.14). The 'bench drill method' yielded high quality DNA, showing rDNA bands. QIAGEN kit yielded very low concentrations of DNA. The 'beadbeater method' was too intensive, shearing DNA. Experiments varying the intensity and length of beadbeating showed that DNA shearing could not be decoupled from quantity of lysed material and quantity of DNA extracted (i.e. more cells broken and DNA, extracted more DNA shearing). As a result DNA extracted via beadbeater method despite cleaning, formed translucent dark brown pellets which did not redissolve well and contained poor quality DNA.

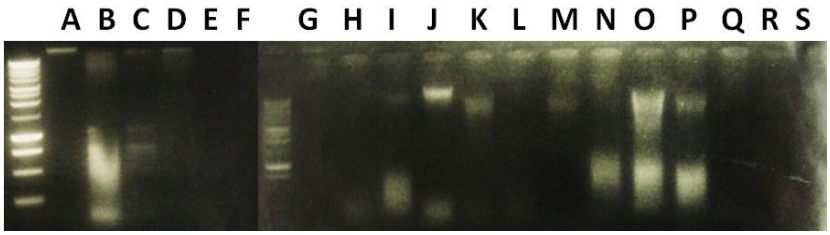


Figure 5.14: DNA extraction using various methods, tested on *Cv* (*Chlorella vulgaris*), *Ce* (*Chlorella emersonii*), *Sv* (*Scenedesmus vacuolatus* 'VT-1').
Each triplicate group of methods follows this species order. (A,B,C) bench drill and standard protocol, (D,E,F) QIAGEN, DNeasy Plant mini kit protocol (G-S) Fastprep120 beadbeater with different matrices (G,H,I) matrix 'C', (J,K,L) matrix 'A', (M,N,O) matrix 'E', (P,Q,R) matrix 'F'.

Despite the high quantity of DNA extracted using the 'bench drill method' PCR of algal DNA required optimisation of PCR conditions and DNA concentration. The presence of DNA smearing (inability to see clear ribosomal bands) suggested a high level of cellular material still present, sticking DNA together. A recommendation from Danson (2009) suggested the addition of 24:25:1 phenol: chloroform: isoamylalcohol (a common alteration to DNA extraction methods used in extracting DNA from cotton) to further purify DNA from 'sticky' contaminants such as glycoproteins (Danson, 2009). When extracting algal DNA for PCR it is requires purification to remove contaminants which may interfere with replication (Mutanda *et al.*, 2011). Due to concerns surrounding quantity of algal DNA extracted, 5µl of DNA was initially used for all 25µl PCR reactions. Serial dilutions of algal DNA showed a marked improvement in PCR products (Figure .5.15). A 18S rDNA gene fragment size of ~1.5kb was expected.

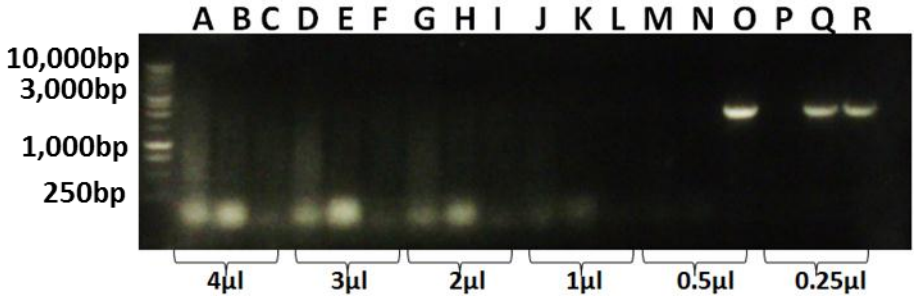


Figure 5.15: PCR products of U18SF and U18SR primers performed with serial dilutions of algal DNA (extracted using the bench drill method).
Each triplicate group of methods follows this species order: *Cv* (*Chlorella vulgaris*), *Ce* (*Chlorella emersonii*), *Sv* (*Scenedesmus vacuolatus* 'VT-1'). (O) *S. vacuolatus*, (Q) *C. emersonii* and (R) *S. vacuolatus*. Fragment size ~1,700bp.

For *C. emersonii* and *S. vacuolatus* dilutions equivalent to 0.5-0.25µl of stock was sufficient for successful PCR. *C. vulgaris* required further dilution to 0.0625-0.03125µl (Figure 5.16).

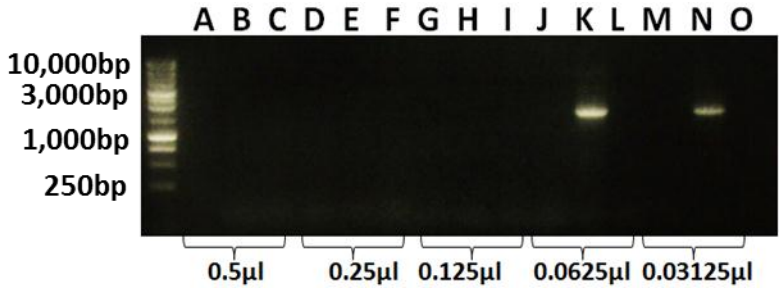


Figure 5.16: PCR products from the amplification of 18S rDNA (using U18SF and U18SR primers) using a DNA dilution series for *Chlorella vulgaris* DNA.
Three repeats were performed of each dilution. Fragment size ~1,700bp.

5.2.5 Primers and identification of isolates

Representative images of isolates from the Roman Baths were taken alongside our reference alga *Chlorella emersonii* (Figure 5.17). Types of algae isolated included; two green algae (B, C), a diatom (D) and four cyanobacteria (E, F, G, H). Isolates B, C, D and E were isolated using the 'filter stack method' and isolates F, G and H were from scrapings on site. The isolated cultures were unialgal prior to DNA extraction and 16S/18S 'barcoding'. Isolates were identified as follows and will occasionally be referred to by their abbreviations (in brackets); B: *Coelastrella saipanensis* (*Cs*), C: *Klebsormidium* sp. (*K* sp.), D: *Hantzschia* sp. (*H* sp.), E: *Choococcidiopsis thermalis* (*Ct*), F: *Microcoleus chthonoplastes* (*Mc*), G: *Mastigocladus laminosus* (*Ml*) and H: *Oscillatoria sancta* (*Os*).

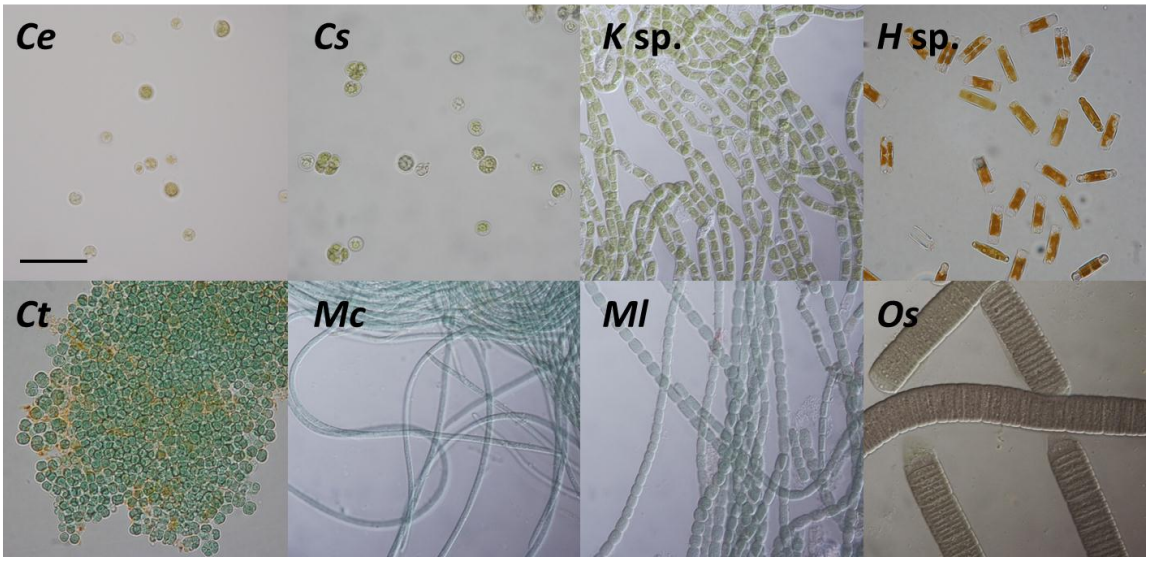


Figure 5.17: Microscope images of algae isolated from the Roman Bath.
All species were isolated from Great Bath unless otherwise stated (*= isolated from Kings Bath) *Chlorella emersonii* (*Ce*), *Coelastrella saipanensis* (*Cs*), *Klebsormidium* sp. (*K* sp.), *Hantzschia* sp. (*H* sp.), *Choococcidiopsis thermalis* (*Ct*), **Microcoleus chthonoplastes* (*Mc*), **Mastigocladus laminosus* (*Ml*), *Oscillatoria sancta* (*Os*). Bar = 20µm.

Microalgae are often 'elastic' in their ability to alter their morphology based on their growth conditions. In a unique environment such as the baths, algae may potentially look different to species standards. In addition, it is not uncommon for algae which are distantly related to look similar and vice versa. As such identifying algae based on morphology can be very challenging. Therefore 'DNA barcoding' was the preferred method for identification of isolated photosynthetic microorganisms. Primers used for barcoding of each isolate were constructed as described in Chapter 2.1.9. Sequences were checked manually and corrections made using Sequencher, sequences were assembled using CLC sequence viewer. Final sequences for all isolates are given in Appendix D.

Isolates were assigned species or genus level identification using Basic Local Alignment Search Tool (BLAST) using the 'total score' values and in some cases images from online culture collections to confirm matches (see methods). In order to be consistent 'Total score' from the BLAST outputs was used as a means of identification (Table 5.5). For any isolates with multiple hits of the same score, visual identification was used to help confirm an ID (using images from online culture collections).

Table 5.5: BLAST search results used for the identification of the seven Roman Bath isolates.
Final identification was primarily based on 'Max Score' yet was confirmed in some cases by the use of morphological data (date accessed 17.01.2012).

Isolate	Abbr	Division	GenBank match ref.	Coverage %*	Max Ident % **	Max Score ***	Other
*Coelastrella saipanensis**	*Cs*	Chlorophyta	AB055800	100	99	3090	Morphology
Klebsormidium sp.	*K* sp.	Chlorophyta	FR717537.1	98	99	1929	Morphology
Hantzschia sp.*	*H* sp.	Bacillariophyta	-	-	-	-	Morphology
Choococcidiopsis thermalis	*Ct*	Cyanophyta	AB039005.1	100	99	2488	
*Microcoleus chthonoplastes**	*Mc*	Cyanophyta	EF654089.1	96	91	1844	
Mastigocladus laminosus	*Ml*	Cyanophyta	AB607204.1	95	99	2385	
Oscillatoria sancta	*Os*	Cyanophyta	AF132933	96	99	1522	

*% of sequence covered by database hit, **% similarity of sequence covered by hit, ***the score is calculated from the sum of the match rewards and the mismatch (independently for each segment).

The isolate identified as *C. saipanensis* with a BLAST search, received identical scores for *Coelastrella saipanensis* and *Ettlia texensis*. Upon closer examination both sequence entries in the database were identical. Images of *Coelastrella* sp. in online collections showed similar

cell granularity ('speckled' inclusions) to the isolate but did not show aggregates of diving cells inside mother cells (a feature of *Ettelia* sp.) and as such was identified as *C. saipanensis*.

For *K*. sp. there were many similarly scored species of the genus *Klebsormidium*. There were no distinct physical features present to identify a species based on morphology, this is a difficulty reported in the identification of species from this genera. *Klebsormidium* spp. a taxonomically diverse and complex genus often found in urban environments (more so on moist surfaces or humid environments with some aquatic species). Despite being one of the most geographically widespread chlorophytic microalgae in the world, taxonomic relationships within the genus are poorly understood, due to its simple morphology and sparse molecular data (Rindi *et al.*, 2008).

For isolate *H* sp. there were many very similarly scored matches from different groups of diatoms including *Nitzschia* sp. which morphologically is very different to our isolate. Diatoms often have species specific morphology of the cell wall, which unlike other groups of microalgae improve identification on morphology alone (Bozarth *et al.*, 2009). This does however require expert experience and as such images were sent to University of Colorado for identification.

For *M. chthonoplastes* most of the outputs in the BLAST search (regardless of sorting criteria) had a ~98% query coverage from *Microcoleus chthonoplastes* strains (with 'maximum identities' of 91%) and other uncultured clones. However, *Geitlerinema* cf. *acuminatum*, had 77% query coverage but 97% max ident. Little has been published on *Geitlerinema* sp. but a few images from culture collections do more closely match the growth morphology of the isolate than *Microcoleus* (i.e. *M. chthonoplastes* isolate grows as an amorphous tangle of filaments like *Geitlerinema* as opposed to discrete bundles of fibres like *Microcoleus* spp.). *M. chthonoplastes* forms large tightly woven bundles of fibres enclosed in a single sheath. However, many strains in culture collections do not share this morphology (Garcia-Pichel *et al.*, 1996), which may be due to incorrect identification or genuine differences in morphology. In order to be consistent 'Total score' from the BLAST outputs was used as a means of primary identification and for any isolates with multiple hits of the same score, visual identification was used to help confirm an ID. As a result this particular isolate has been identified as *M. chthonoplastes*.

Five of the seven nucleotide sequences from the Roman Bath isolates have been accepted by the NCBI onto the database GenBank and have been assigned accession numbers as follows: *Coelastrella saipanensis* (JX316760), *Choococcidiopsis thermalis* (JX316763), *Hantzschia* sp. (JX316762), *Klebsormidium* sp. (JX316761), *Mistogocladus laminosus* (JX316764). *Oscillatoria sancta* and *Microcoleus chthonoplastes* were not accepted by the NCBI GenBank database due to duplications within the sequence and a high incidence of gaps and internal N's (unidentified nucleotides).

5.2.6 Culture maintenance

Maintenance of stocks cultures can heavily depend on the strain (Dworkin *et al.*, 1992). Stock cultures of isolates were stored in their preferred medium (BG-11 for cyanobacteria, BBM for green algae and Diatom Medium (DM) for *Hantzschia* sp.) at sub-optimal temperatures (~15°C) and low light (lab bench). These were routinely shaken and subcultured. Cryogenic sample preparation (as described by Andersen, 2005) proved unsuccessful (with *C. emersonii*), yet should be implemented to prevent genetic drift of isolates (Mutanda *et al.*, 2011). Low light levels caused a notable change in the morphology of *O. sancta* (Figure 5.18).

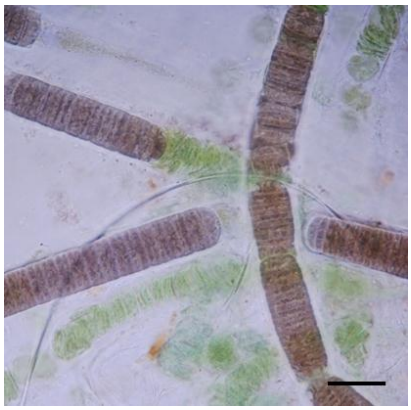


Figure 5.18: Light microscope images of *O. sancta* filaments undergoing a change in morphology when cultivated under low light, from brown filaments to amorphous green cell stacks.
Bar = 20μm.

O. sancta showed a change in structure from organised brown filaments to amorphous green cells. It has been described that *Oscillatoria* sp. has a preference for low light conditions (Kruskopf and du Plessis, 2006). It has also been reported that in *Oscillatoria* sp. increases in temperature can drastically change the colour and productivity of a culture (Gribovskaya *et al.*, 2007).

All 7 isolated microalgal strains from the Roman Baths, were deposited at the CCAP on the 15.05.12 and were assigned the following accession numbers; *Coelastrella saipanensis* (CCAP 217/9), *Choococcidiopsis thermalis* (CCAP 1423/1), *Hantzschia* sp. (CCAP 1030/1), *Klebsormidium* sp. (CCAP 335/20), *Microcoleus chthonoplastes* (CCAP 1449/2), *Mistogocladus laminosus* (CCAP 1447/9) and *Oscillatoria sancta* (CCAP 1459/46).

5.3 Discussion

Microalgae are a diverse group of organisms with only a handful currently used in research. This Chapter describes the attempts to isolate and identify microalgae and cyanobacteria from the Roman Baths (Bath, UK). A variety of isolation methods were used including medium enrichment, filtering, agar plate dilution and filter-paper culturing. Biomolecular methods were used for identification, using the 18S (eukaryotic) and 16S (prokaryotic) rDNA gene sequences. Visually assessing the morphology of the isolates with images from online culture collections was used as a supplementary tool to aid in establishing the identity of the species determined from the results of the BLAST search. A total of 7 strains were isolated; 4 cyanobacteria, 2 green eukaryotic algae and 1 diatom.

5.3.1 Isolation of algae from environmental samples and decontamination

Excavation of Baths began in 1878 and therefore has only been open to the elements for 130 years (Byrne, 2008). It is likely that all the species present have been introduced by weathering, movement of water and local biota. It is important to note that in the Roman Baths microbial mats there was a dominance of cyanobacteria, which could be due to warmer temperatures, lower light or presence of particular chemicals. Once established, cyanobacteria and algae can influence the growth characteristics of other microorganisms. For example, *Oscillatoria* sp. is documented to release a phycoerythin-like protein, which inhibits the growth of green algae but not other bacteria and cyanobacteria (Karseno *et al.,* 2009). The isolates *O. sancta*, *M. chthonoplastes*, *M. laminosus* and *H.* sp., were found to be the predominant species and were repeatedly found when attempting to isolating others.

Cyanobacteria are good candidates for removal of compounds from waste streams as they produce sufficient amounts of biomass, of which the composition can be manipulated by altering environmental and operational parameters (Markou and Georgakakis, 2011). Yet they may not be suitable for biodiesel production, as no oleaginous bacteria have been described to date (Park *et al.,* 2005). *K.* sp., *M. chthonoplastes, M. laminosus and O. sancta,* are filamentous and therefore desirable for mass culturing due to their suitability for filtration, a cheap low energy method of dewatering (Gaur *et al.,* 1960). *M. laminosus* is a nitrogen fixing species as it contains heterocysts (specialised non-photosynthetic cells which fixate nitrogen). Both of these features could potentially lower economic costs and give a more viable production process on scale-up (Christenson and Sims, 2011).

There is little information regarding isolation and cultivation of thermophilic cyanobacteria. Sources have suggested that single cell isolation has limited success (Castenholz, 1969), as bacteria are often found to be closely associated with the muciferous layer of the algal cell walls. Micromanipulation, filtration and use of chemicals may only be successful with some algal groups (Ferris and Hirsch, 1991), and antibiotics typically have a narrow concentration range as they are often lethal to microalgae (Issa, 1999). Dilution and plating in this case was found to have had limited success, as impurities in the agar (a substance derived from algae) are inhibitory to the growth of some algae and encourage growth of bacterial and fungal contaminants. It is likely that the reason why some cyanobacterial isolates grew poorly on agar is that there was something present that is toxic to them rather than nutrient deficiency (Dworkin and Falkow, 2006). Sometimes the timeframe between formation of algal colonies and plates being contaminated can be short and this was a large issue in the early stages of bioprospecting the Roman Baths. The inability for isolates to grow in filter-stacks of autoclaved Roman bath water may be due to the absence vital nutrients, synthesized by existing in a microbial mat community. Bacteria can greatly improve growth of algae by affecting pH, releasing inorganic nutrients, N-derivatives and increasing CO_2 availability (Banerjee *et al.*, 2002).

Scrapings of dominant species and filtration of vast quantities of water was successful in isolating algae from the baths. The discovery of older methods for decontamination ('plate scoring' and 'filter stack' methods) was invaluable to decontamination of isolates within the scope and budget of this project. Although most of the contaminants have been removed and some isolates may be axenic, the isolates have been described as unialgal. From the initial sampling experiments, many more algal species are estimated to be present in the bath, if only at a low density.

5.3.2 Identification of microalgae

In order to amplify and sequence 16S (cyanobacterial) or 18S (eukaryotic) rDNA genes, the isolates needed to be subjected to some decontamination. However, it is evident that the microbial mat communities present in the Roman Baths were required (to some extent) for microbial growth and survival. Hence it is highly likely that resident algae had formed relationships with other organisms present (Croft *et al.*, 2006). Microbial mats are often important primary producers in extreme habitats, playing an important role in biochemistry

stabilisation, providing a more suitable environment in which species can better survive (Pattanaik, 2008).

The lysis of algal and cyanobacterial cells proved a challenge. The beadbeating method of lysing cells (successful in breaking tough cells) was coupled with shearing of DNA (resulting in translucent brown pellets which did not redissolve readily in water and produced smears when ran on a gel). DNA extraction was optimized using the bench drill method and the addition of phenol:chloroform:isoamylalcohol (25:24:1), which precipitated a surprising quantity of contaminant biomaterial with resultant high quality DNA. Serial dilutions of algal DNA showed that much lower quantities were needed for successful PCR. This may have been due to presence of remaining cellular components, sticking DNA strands together making it inaccessible to primers and polymerases.

Currently, using U16S and U18S rDNA gene sequences and the NCBI BLAST search as a means of identification is fraught with problems. There are currently few gene sequences available online, most of which are largely unregulated, with a bias toward more commonly used species. There are also disputes as to which fragments of DNA to use for identification. Cox1 is the standard for most 'higher animals' yet not suitable for green algae and higher plants (because rate of evolution of this region is too slow). Common regions used for algal identification are: rRNA genes, mitochondrial genes and plastid genes. Yet the search is on-going to find a single universal short DNA fragment which gives a clear identification of species (Surek, 2008). Geographical location can also confer differences in these gene sequences *within* a species; this has been demonstrated with *M. chthonoplastes* (Garcia-Pichel *et al.*, 1996) and *Synechococcus* sp. (Miller and Castenholz, 2000), which are often dominant species in intertidal microbial mat communities (Pattanaik, 2008). These types of analyses are very useful and have provided evidence to suggest that more thermotolerant lineages evolved from less thermotolerant ancestors. However, this coupled with the concerns listed above, can make some species hard to identify using DNA barcoding.

Morphological identification when applicable, is not only difficult but can be misleading. Many microalgae lack distinct morphological features, making them hard to identify (Pulz and Gross, 2004) and difficult to tell apart even by experts (Belcher and Swale, 1976). Cells can also change shape and size during their lifecycle and under certain conditions. For algal identification and taxonomy a 'polyphasic' approach is often needed, using both molecular

and morphological data (Surek, 2008). Due to the nature of the identification methods used and poor regulation and sequence data availability, it is unclear how 'unique' these isolates are, compared to those found in culture collections.

Identification of the Roman Bath isolates attempted to be consistent and robust, yet encountered multiple problems. Identification of isolates therefore may be incorrect, due to the lack of available genomic data and potential incorrect data in online databases and culture collections. For the purposes of this project the identified algae isolated from the Roman Baths were as follows: *Coelastrella saipanensis*, *Klebsormidium* sp., *Hantzschia* sp., *Chroococcidiopsis thermalis*, *Mastigocladus laminosus*, *Microcoleus chthonoplastes* and *Oscillatoria sancta*. Poor sequence data from *Microcoleus chthonoplastes* and *Oscillatoria sancta* is likely due to errors in replication from using 'Dreamtaq' rather than utilising a polymerase which can proof-read sequences and repair errors. The sequence quality could have also been improved by using multiple sequencing and deriving an average for ambiguous nucleotides.

5.3.3 Further work

It should be noted that there is an abundance of microalgae present in the baths, more than have been described here. It would be of interest to continue bioprospecting using the filter stack method. Perhaps additionally, selective pressures could be applied, for example using other types of media such as waste water. It would also be interesting to discuss all the identification methods and results with an expert to have feedback on the species names ascribed to my isolates.

5.3.4 Summary

The Roman Baths contain a great diversity of microalgae, the majority of which are cyanobacteria. The microbial mat communities which exist in the roman baths no doubt provides a survival advantage, to microorganisms. Cyanobacteria are good candidates for biotechnological investigation, yet may not be suitable for biodiesel production, as no oleaginous bacteria have been described (Park *et al*., 2005). *K.* sp., *M. chthonoplastes*, *M. laminosus and O. sancta*, are filamentous, desirable for mass culturing due to their suitability for filtration, a cheap low energy method of dewatering (Gaur *et al.,* 1960). *M. laminosus* is a nitrogen-fixing species (containing heterocysts), which could lower the demand for nitrates

giving a more viable production process on scale-up (Christenson and Sims, 2011). Isolation of microalgae from environmental samples is a challenging process (requiring the use of multiple techniques), matched only in difficulty by the challenge of identification. Identification of microalgae using molecular techniques can only be as successful as the resources available, which are currently underdeveloped and largely unregulated.

6. TEMPERATURE TOLERANCE AND FATTY ACID METHYL ESTER ANALYSIS OF ROMAN BATH ISOLATES

6.1 Introduction

6.1.1 Cost of microalgal biofuel - the ideal isolate

In time, genetic engineering of commonly studied species may produce highly productive strains, yet problems with containment may prohibit their use in industry (Chapter 3.1). As such, bioprospecting for naturally occurring highly productive or unusual species, must be continued alongside developments in microalgal genetic tool advancements.

The ideal biodiesel alga should achieve high cell densities, have a rapid growth rate and low nutrient requirements, is easy to dewater and break open and most importantly has a high oil content. The ability for a particular species to withstand particular culture conditions may make it preferable over another candidate, even if there is a drop in productivity. For example an open pond system (perhaps for scrubbing wastewater) could benefit from using an extremophile in order to easily maintain a monoculture, yet this could be coupled with a lower doubling rate. Even small changes such as these could reduce costs and still make the overall process lucrative (Ali, 2011). The protection such an 'extreme' environment offers from contamination and domination of a culture by invasive local algae or contaminants, can sometimes outweigh any slightly negative traits (Ali, 2011).

In highly irradiated, hot regions (e.g. Australia) or culture systems utilising hot flue gas as a CO_2 source, cooling of the cultures is likely to become a critical parameter of the process. On cultivating microalgae outdoors, cultures are subject to large temperature swings and possible invasion from other species. Therefore an ability to survive higher temperatures, if not to flourish at them, is an extremely desirable trait (Pulz and Gross, 2004). In fact the few species which are successfully cultivated commercially in open ponds are extremophiles, able to grow in a highly selective environment (Xu *et al.*, 2009). Open ponds and closed systems can be prone to temperature spikes, which have the potential to wipe out entire cultures of algae (Barsanti and Gualtieri, 2006). Even in temperate regions, seasonal and daily fluctuations are still capable of wiping out entire cultures, as temperatures can reach as high at 30°C above ambient in a closed PBR without cooling equipment (Kunjapur and Eldridge, 2010). Although algae may have a wide growth range optimal growth temperatures are usually in a narrow range specific to each strain (Kunjapur and Eldridge, 2010). Temperature control in algal

systems increases the energy demand of culturing (Morweiser *et al.,* 2010). An ability to tolerate if not thrive in a warm environment would therefore be beneficial.

Selective pressures and changing environmental conditions mean a range of microalgae can be found in extreme environments worldwide (Mutanda *et al.,* 2011). Traditional regions to investigate for novel algae to be used commercially are hypersaline environments and thermophilic springs. These robust habitats may offer algae better adapted to specific conditions and may increase the chances of isolating unique hyper-lipid producing microalgae.

6.1.2 Screening for the 'ideal' oleaginaceous candidate

The majority of microalgae sourced from thermophilic regions are cyanobacterial. There has been substantially more work published on eukaryotic algal lipids (particularly *Chlorella* spp.). However despite the lack of oleaginaceous cyanobacteria (Parmar *et al.,* 2011), they have been found to produce lipids, most of which are saturated with a large proportion of C16:0 and C18:0 (Karatay and Dönmez, 2011). Eukaryotic algae accumulate lipid as storage material in the cytosol when under stress, whereas cyanobacteria accumulate lipids in thylakoid membrane suggesting they may be capable of accumulating lipids during photosynthesis and faster growth rates (Karatay and Dönmez, 2011).

Rapid species screening for valuable products is by no means straightforward. Traditional solvent extraction with analysis by GC or HPLC is disadvantageous due to poor extraction levels, large quantities of biomass and time required. Nile red staining can be used to determine the neutral lipid content of microalgal cells and is useful as a high throughput screening method, when combined with fluorometric methods for new species or mutants (Elsey *et al.,* 2007). Nile red can also be used as a means of detecting PHA biopolymers, synthesised by many cyanobacteria (Chapter 1.2.2). Absolute measurements can be inferred by generating a calibration curve for fluorescence and lipid content determined by gravimetric methods or with standards (Elsey *et al.,* 2007)

However, Chapter 3.2.2 already demonstrated the variability in stain uptake of different species of algae depending on their cell wall thickness and morphology, and as such staining may be an unreliable method for detecting lipids or PHAs. This also applies to valuable by-products (such as vitamins). However other screening methods may require more time and

development. One solution is to genetically characterise isolates for known genes which infer presence of a product. For example, *pha*C has a high homology in PHA accumulating cyanobacterial strains but low homology in non-accumulating strains, rendering PCR product analysis a viable means of assessing ability to produce the biopolymer (Miyake *et al.*, 2000). However, the most reliable and commonly used screen for analysing a variety of unstudied algal species, remains a lengthy solvent extraction method and analysis by GC-MS.

6.2 Results

6.2.1 Growth at different temperatures

A preliminary growth experiment was carried out to determine a suitable low, medium and high temperature values at which to conduct further experiments on the Roman isolates. This was necessary due to limited resources (space, equipment and time) and the biomass required for product analysis. The preliminary experiments were conducted in 15ml tubes using heat blocks in order to examine a wide range of temperatures for all species with replicates (216 samples). Results of the preliminary temperature tolerance experiments (Table 6.1) showed that eukaryotic algae (*C. emersonii*, *C. saipanensis*, *K.* sp. and *H.* sp.) behave similarly and are able to grow at 30°C but prefer lower temperatures of 20-25°C. The cyanobacterial isolates had more variation in temperature tolerance between them, yet all showed growth at 35°C (with *M. laminosus* and *M. chthonoplastes* showing growth up to 45°C), unlike the eukaryotes.

Table 6.1: Preliminary temperature tolerance experiments comparing growth of *C. emersonii* and Roman Bath isolates.
Growth was assessed visually by comparing samples across the temperature ranges.

Species	20°C	25°C	30°C	35°C	40°C	45°C	50°C	55°C	60°C
C. emersonii	++	+++	++	+	-	-	-	-	-
C. saipanensis	++	+++	++	+	-	-	-	-	-
Klebsormidium sp.	++	+++	++	+	-	-	-	-	-
Hantzschia sp.	++	NT	++	NT	-	NT	NT	NT	NT
C. thermalis	++	NT	++	NT	++	NT	NT	NT	NT
M. chthonoplastes	+	+	++	+++	+++	++	-	-	-
M. laminosus	+	+	++	+++	+++	++	-	-	-
O. sancta	++	++	++	++	+	+	-	-	-

– no growth, + poor growth, ++ good growth, +++ vigorous growth, NT not tested.

Based on these results, further growth experiments were carried out at 20°C, 30°C and 40°C, to give a low, medium and high value for comparison. The growth rate and effects of nitrogen starvation were studied over 12 days at these temperatures (Figure 6.1, all other data is presented in full in Appendix F). The effects of nitrogen starvation have been widely studied in algae, often triggering hyper-accumulation of lipids in eukaryotic species and accumulation of a variety of storage products in cyanobacteria (Cha *et al.*, 2011).

Temperature tolerance of species cultured in 100ml in open flasks supported preliminary data, whereby eukaryotic algae accumulated maximal biomass between 20-30°C yet were

incapable of growth at 40°C (Figure 6.1). *K.* sp. and *C. saipanensis* accumulated the most biomass at 30°C, comparable to the reference alga *C. emersonii*. As for the cyanobacteria, single-celled *C. thermalis* accumulated more biomass at a lower temperatures of 20°C, whilst *M. chthonoplastes, M. laminosus* and *O. sancta* acquired the most biomass at 30°C, with *M. chthonoplastes* and *M. laminosus* showing better growth at 40°C than 20°C. This was not found to be the case for *O. sancta* whose growth was impaired at 40°C. *M. laminosus* acquired the most biomass of all the cyanobacteria, at a temperature of 30°C (Figure 6.1).

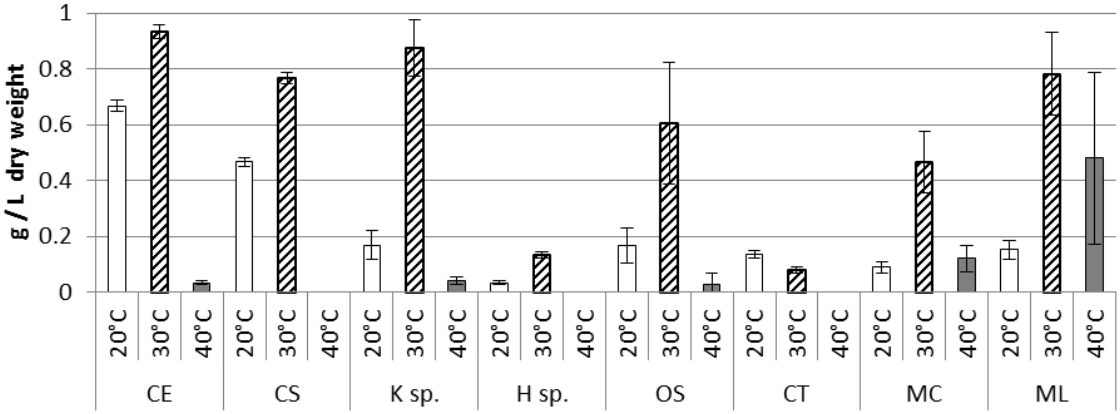


Figure 6.1: Growth of *C. emersonii* and Roman Bath isolates in nitrogen sufficient medium (in g l^{-1} after 12 days).
No repeats were performed due to spatial constraints.

Of all the algae tested, *C. emersonii* accumulated the most biomass at 20°C (0.7 g l^{-1}) Both *C. emersonii* and *K.* sp. accumulated the most biomass at 30°C (0.9 g l^{-1}) and *M. laminosus* the most biomass at 40°C (0.5 g l^{-1}). Both *H.* sp. and *C. thermalis* were very productive at any of the temperatures investigated (<0.2 g l^{-1}) (Figure 6.1). It must be noted that at 30ºC and 40ºC it took *C. thermalis* and *H. sp.* ~7days before' healthy' (cells of good size and colour) growth is observed, suggesting an 'adjustment period' is required under these conditions. Also at 40ºC some cells of *C. emersonii* were present as aggregated translucent cysts.

6.2.2 Staining for lipids

Staining was used to try and visualise lipids, other products or significant changes to morphology. Cyanobacteria for example, have been reported to synthesise insoluble granules of polyhydroxyalkanoates (PHAs, a type of biological polymer) in the cytoplasm, which could become a valuable commodity. PHAs are not only detectable by H-NMR (Shivastav *et al.,* 2010) but also by staining with lipophilic dyes, such as sudan black and nile red

(Gouviea *et al.*, 2009), producing dark blue or fluorescent staining (Chen *et al.*, 2011). To visualise lipids and any PHAs nile red and 'BODIPY' were used. Nile red has different emission properties based on the polarity of lipids, with non-polar/saturated lipids staining red, and polar/unsaturated lipids staining yellow (Elsey, 2007). BODIPY is a relatively new green lipophilic fluorescent dye, often used to sort cells with high oil content (Cooper *et al.*, 2010).

Staining gave a visual indication of the lipid products contained in a sample (Figure 6.2, for all samples see Appendix E). Photosynthetic pigments in eukaryotic algae and cyanobacteria autofluoresce under the same excitation wavelength as nile red, which can make it difficult to identify stained regions. However the fluorescence of these pigments is useful in assessing the health of the cell, as stressed cells undergo breakdown of these pigments. This can be seen during nitrogen starvation of *Klebsormidium* sp. at 20°C (Figure 6.2a and 6.2b). At 20°C *K.* sp. (Figure 6.2a and 6.2b) had clearly visible lipid droplets and a noticeable lack of chlorophyll. At 30°C however, during nitrogen starvation, *K* sp. showed little or no breakdown in chlorophyll or accumulation of lipids (Figure 6.2c and 6.2d).

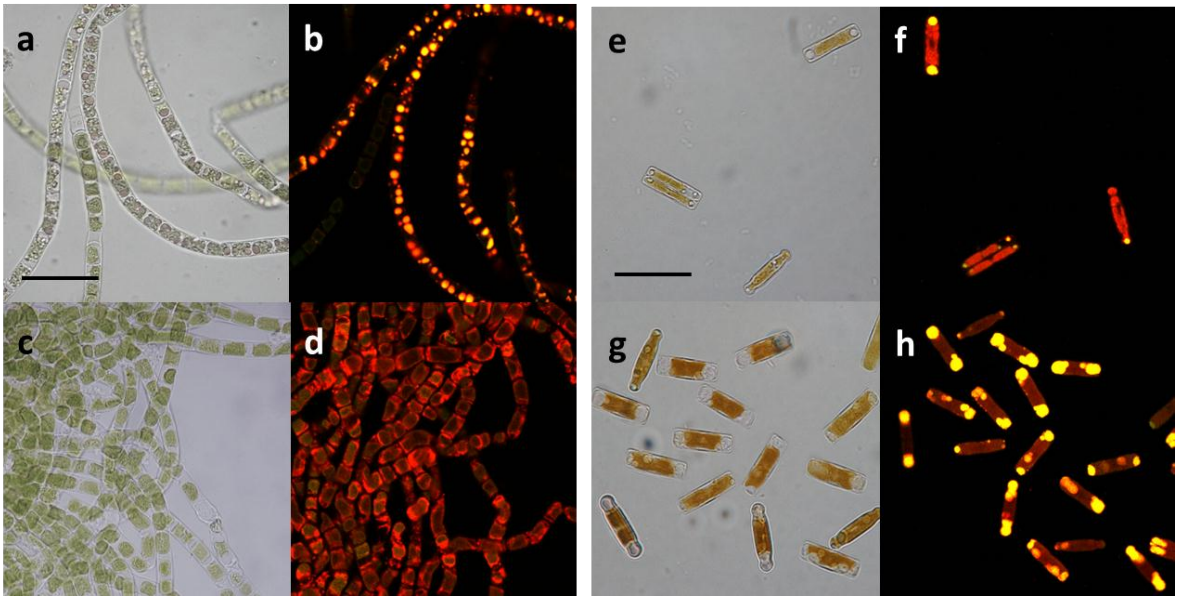


Figure 6.2: Microscope images of *Klebsormidium* sp. cultivated at 20°C (a,b) and 30°C (c,d) and starved of nitrogen. Microscope images of *Hantzschia* sp. cultivated at 30°C with sufficient nitrogen (e,f) and starved of nitrogen (g,h).
Lipid droplets can be seen as fluorescent yellow inclusions stained with nile red. Viewed under the light microscope and confocal. Bar = 20µm.

Nitrogen starvation usually encourages accumulation of lipids in most microalgae. *H.* sp. at 30°C showed higher accumulation of lipids under nitrogen sufficient conditions (Figure 6.2 F) compared to nitrogen starved conditions (Figure 6.2 H). Poor permeation of nile red and BODIPY into the cells of some species (notably thick walled cyanobacteria such as *C. thermalis* – further discussed in Chapter 3.2.2) rendered staining with fluorescent stains somewhat ineffective to determine presence of storage products. As a result samples of Roman Bath isolates were also subjected to analysis by TEM.

6.2.3 Transmission electron microscopy for the visulaisation of algal storage products

TEM is by no means a quick screening method, but was carried out as a means of better visualising the cell morphology and contents of the Roman Bath isolates, after staining proved very variable and ineffective (in particular for the cyanobacteria *O. sancta*, *M. chthonoplastes*, *M. laminosus* and *C. thermalis*). Fresh cultures of all isolates were cultivated at 30°C (an ambient temperature for all strains) and subjected to nitrogen starvation (to induce product accumulation) prior to preparation and analysis by TEM.

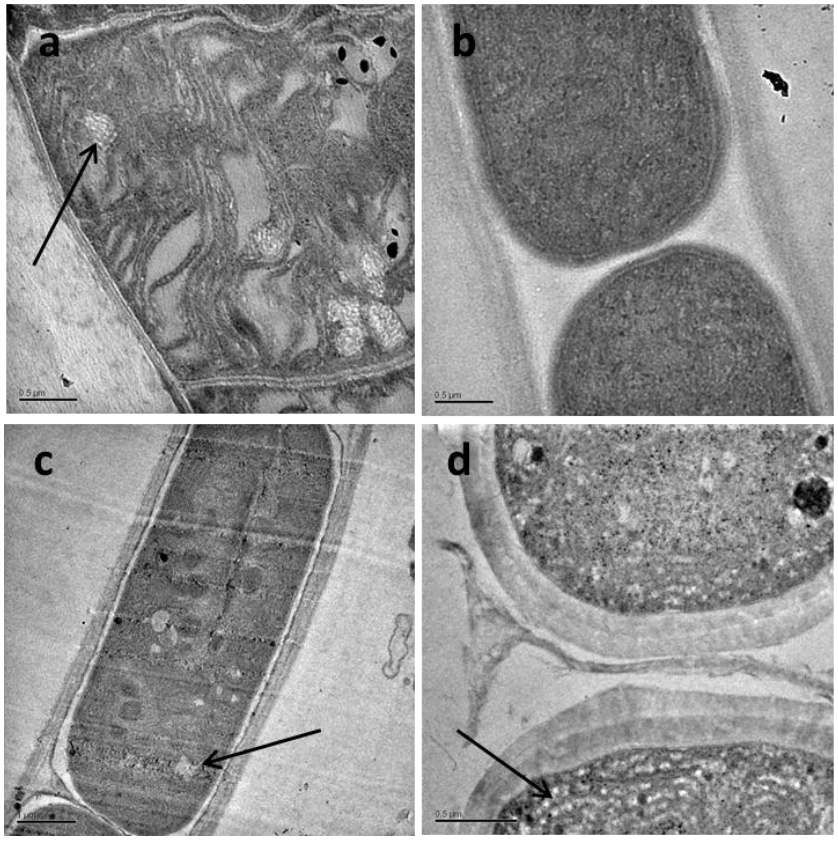


Figure 6.3: TEM images of (a) *O. sancta*, (b) *M. chthonoplastes*, (c) *M. laminosus* and (d) *C. thermalis*. Cultivated at 30°C with nitrogen starvation. Arrows indicate white granules/inclusions. Bar = 0.5µm.

Small white-speckled granules could be seen amongst the thylakoid membranes in *O. sancta, M. laminosus* and *C. thermalis*, but not visible in *M. chthonoplastes* (Figure 6.3). Non-nitrogen starved cultures did not have such a high incidence of these inclusions, indicating that the granules are storage products (potentially lipids or PHAs, which typically accumulate under nitrate deficiency or stress). *K. sp*. and *C. saipanensis*, accumulated significantly more lipid under nitrogen starvation (compared to nitrate sufficient conditions), much like our stock strains of *Chlorella* sp. and *P. ellipsoidea* (Figure 6.4).

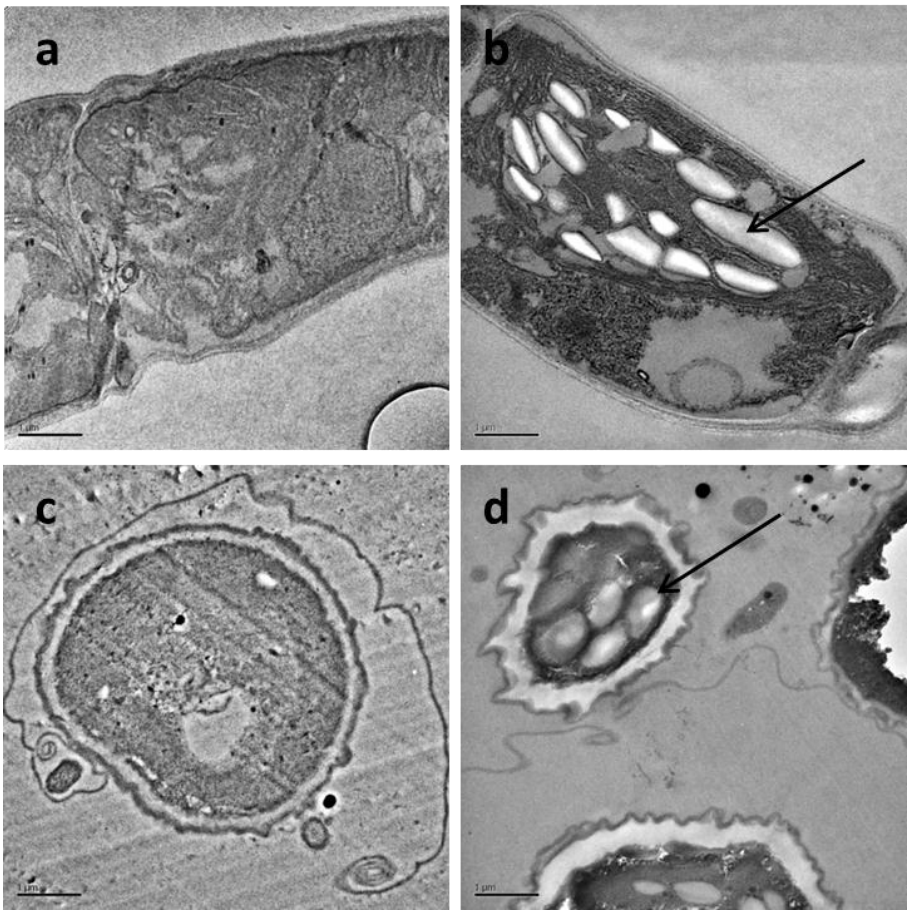


Figure 6.4: TEM images of (a) *K. sp*. (nitrate sufficient), (b) *K. sp*. (nitrate-starved) and (c) *C. saipanensis* (nitrate sufficient), (d) *C. saipanensis* (nitrate-starved).
Arrows indicate pools of stored lipids or starch. Bar = 1µm.

Unfortunately during TEM preparation *H. sp*. did not pellet well, due to low cell densities and the sample was lost. Poor sectioning occurred due to sample resins curing in a moist environment, rendering it brittle. This coupled with the high viewing voltage of this set of TEM samples and prior carbon coating made for granular images. These observations may indicate the presence of PHAs, which were not detected by staining with nile red.

6.2.4 FAME analysis

To assess the effectiveness of the microalgae to produce biodiesel (at different temperatures with and without nitrogen starvation), lipids of the Roman isolates and *C. emersonii* were analysed by GC-MS after being converted into FAMEs though acid catalysed esterification.

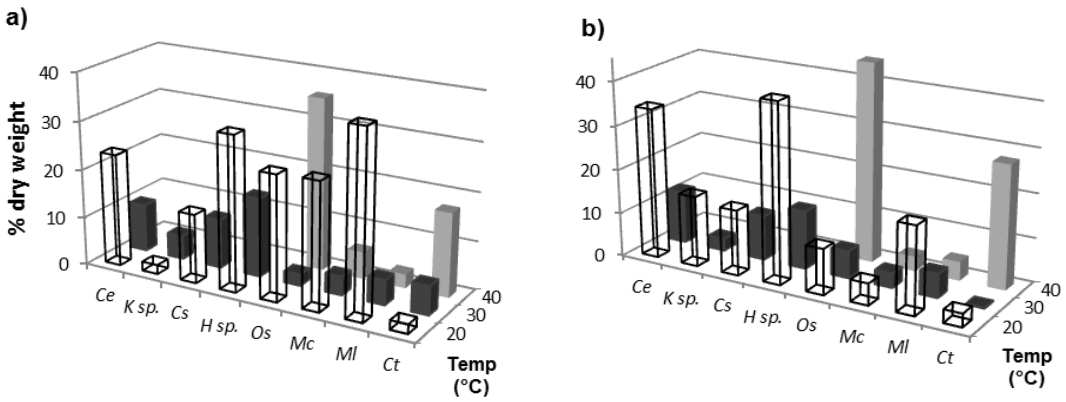


Figure 6.5: Lipid content as a wt% of algal sample for the Roman isolates cultivated under (a) nitrogen enriched and (b) nitrogen starved conditions.
No repeats were performed due to spatial constraints.

Irrespective of any other effect, the green algae produced less lipid at higher temperatures (Figure 6.5). Reducing the nitrogen content generally increased lipid accumulation in the green algal isolates. This effect was particularly predominant in *C. emersonii* where the lipid content increased from 23wt% to 34wt% under nitrogen starved conditions. A similar effect was seen with *K.* sp. & *H.* sp.. However nitrogen starvation did not dramatically affect the lipid content in *C. saipanensis*. As a green eukaryote, it may have been expected to behave similarly to *C. emersonii*.

Alternatively, nitrogen-starvation of cyanobacteria had a detrimental effect on the lipid percentage at both 20°C and 30°C. At 40°C, however, *O. sancta* produced a high percentage (10.4-45.6%) of lipid under most conditions (with the exception of 30°C). This effect was not observed in *M. laminosus*, *M. chthonoplastes* or *C. thermalis* (Figure 6.5).

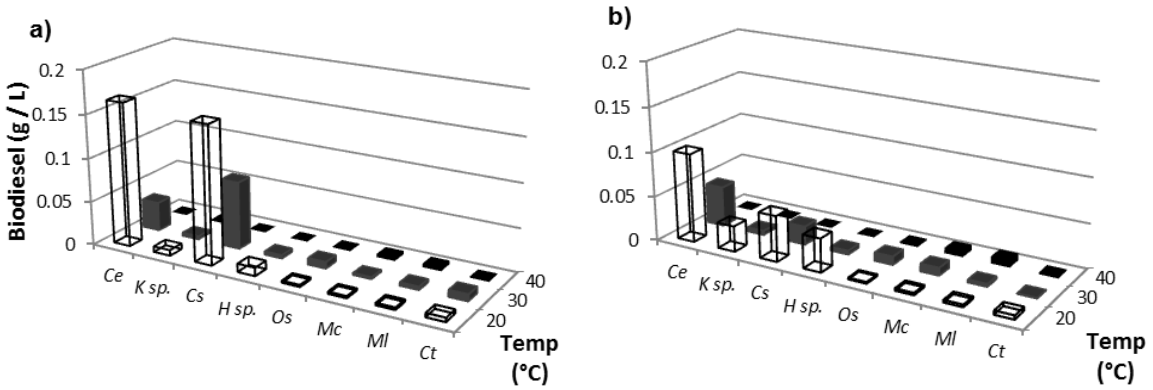


Figure 6.6: Total amount of biodiesel recovered from the Roman isolates cultivated under (a) nitrogen enriched conditions and (b) nitrogen starved conditions.
No repeats were performed due to spatial constraints.

Reducing the nitrogen content in the growth media had a detrimental effect on growth rate, resulting in the need for a two-step process for biodiesel production (growth and nitrogen starvation). The highest biodiesel production was observed for *C. emersonii* and *C. saipanensis* at 20°C under nitrogen enriched conditions (Figures 6.6-6.7), where the lower percentages of lipid produced were amply made up for by a higher growth rate. Little biodiesel was recoverable from *K.* sp. and *H.* sp. under nitrogen enriched conditions at any temperature, yet an increase in lipid content was observed on a reduction in the nitrogen content.

None of the cyanobacteria produced a large amount of biodiesel at any temperature. None of the cyanobacteria produced a large amount of biodiesel irrespective of nitrogen conditions at 20°C and 30°C (Figure 5) compared to green algae *C. emersonii* and *C. saipanensis* (>0.15 g l^{-1} of biodiesel). For the most part, this is due to low levels of biomass accumulation in the cyanobacterial isolates (Figure 6.1). However, with nitrogen reduction a larger amount of biodiesel was recovered from the cyanobacteria at 40°C, though this was still only a small amount of biodiesel overall. All cyanobacteria increased lipid production at 40°C under nitrogen depletion.

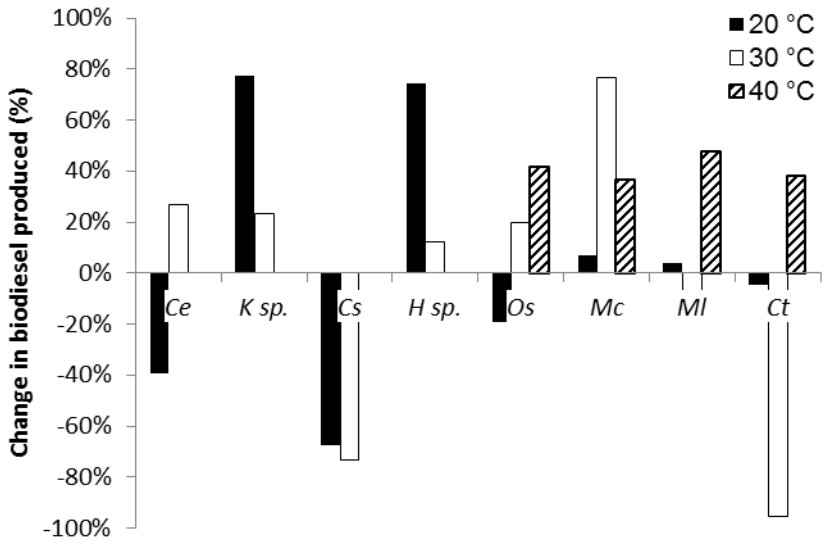


Figure 6.7: Percentage change in the total amount of biodiesel produced upon nitrogen starvation of the Roman isolates.
No repeats were performed due to spatial constraints.

6.2.5 FAME profile

The properties of biodiesel are highly reliant on the FAME profile. Though chain length and the degree of unsaturation do have an effect on the fuel properties, the FAMEs can be grouped into three loose categories (saturated, monounsaturated and polyunsaturated) to predict fuel performance. Fuels rich in saturated esters have better cetane numbers but poor viscosity and low temperature properties, monounsaturated esters tend to have reasonably low temperature properties and viscosity, where fuels high in polyunsaturates have excellent low temperature properties and a low viscosity but poorer cetane numbers and oxidative stability. The full FAME profiles for all samples are given in Appendix F.

On a reduction in nitrogen content for *C. emersonii* the FAME profile did not change significantly (Figure 6.8). However, on an increase in temperature the polyunsaturated components increased (in both nitrogen sufficient and deficient circumstances) predominantly at the cost of the monounsaturated components. This was also found to be true for *C. saipanensis*. Both of these algal species had very similar FAME profiles under all conditions. *K.* sp., on the other hand, was richer in saturates. For *K.* sp., reducing the nitrogen increased the formation of unsaturated FAMEs, with both nitrogen enriched 20°C and 30°C samples being predominantly saturated. Like the green algae *C. saipanensis* and *C. emersonii*, the FAME profile of *H.* sp. did not change significantly with the reduction in nitrogen.

However, on an increase in temperature far more saturated components were formed. The level of saturates in both *K.* sp. and *H.* sp. would have a detrimental effect on the quality of the biodiesel produced from these organisms.

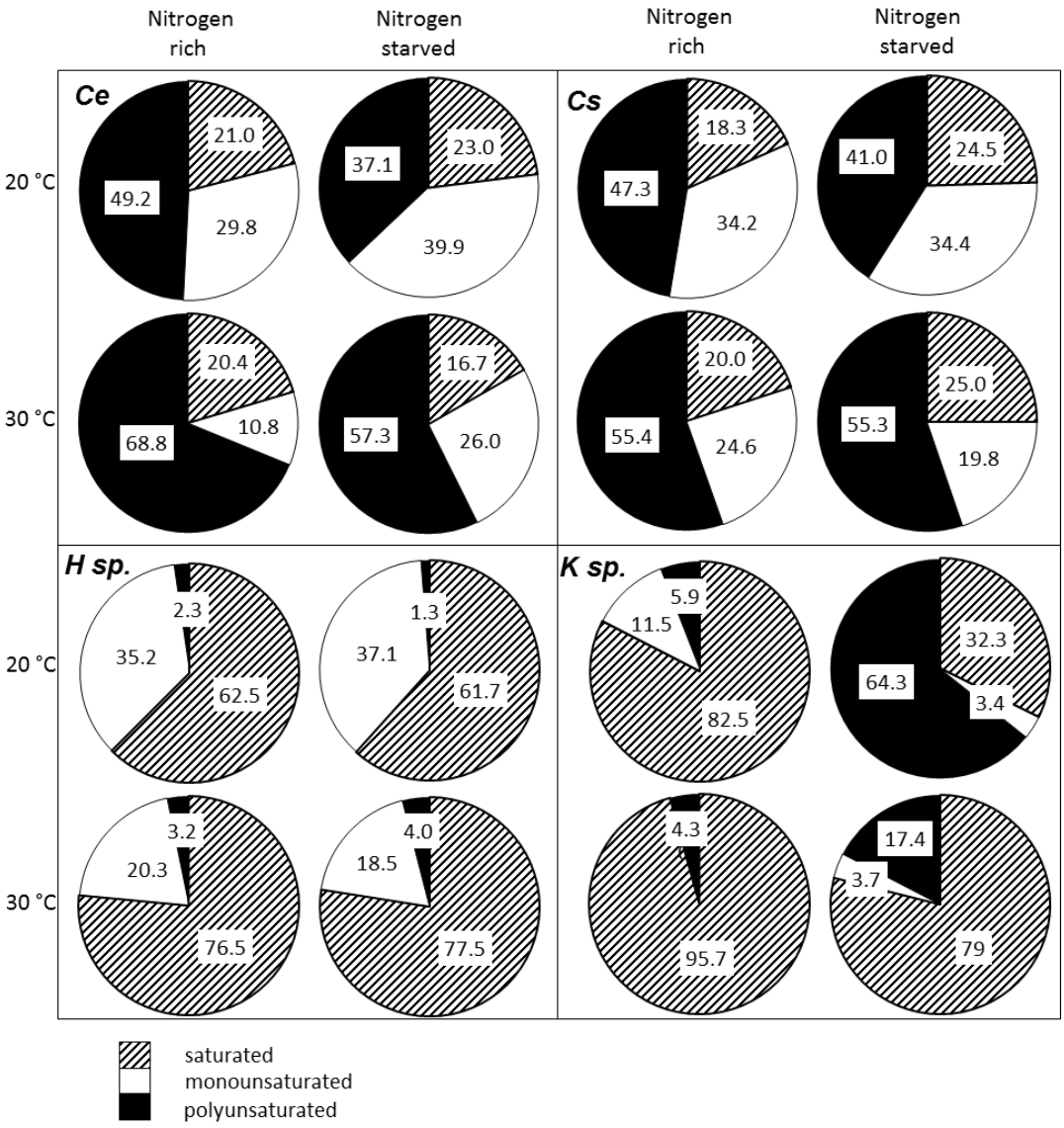


Figure 6.8: FAME profiles of *C. emersonii* and eukaryotic Roman isolates represented as % total FAMEs. This data has been summarised into saturates, monounsaturates and polyunsaturates.

The FAME components of the cyanobacteria were much simpler than their algal counterparts (Figure 6.9). Unfortunately, not enough biodiesel was recovered from *O. sancta* or *C. thermalis* for a complete analysis of the FAME profile (see Appendix F). *M. chthonoplastes* produced only saturated esters at low temperatures (and with sufficient nitrogen at 30°C). On nitrogen depletion (or an increase in the temperature to 40°C), unsaturated esters were formed producing a composition more conducive for use as a biofuel.

M. laminosus produced no polyunsaturated esters under any of the conditions examined. At low temperatures there were around 90% saturates decreasing to roughly 66% when the temperature was increased to 30°C, and just over 50% when the microbes are grown at 40°C. Reducing the nitrogen content had little effect on the composition at any temperature.

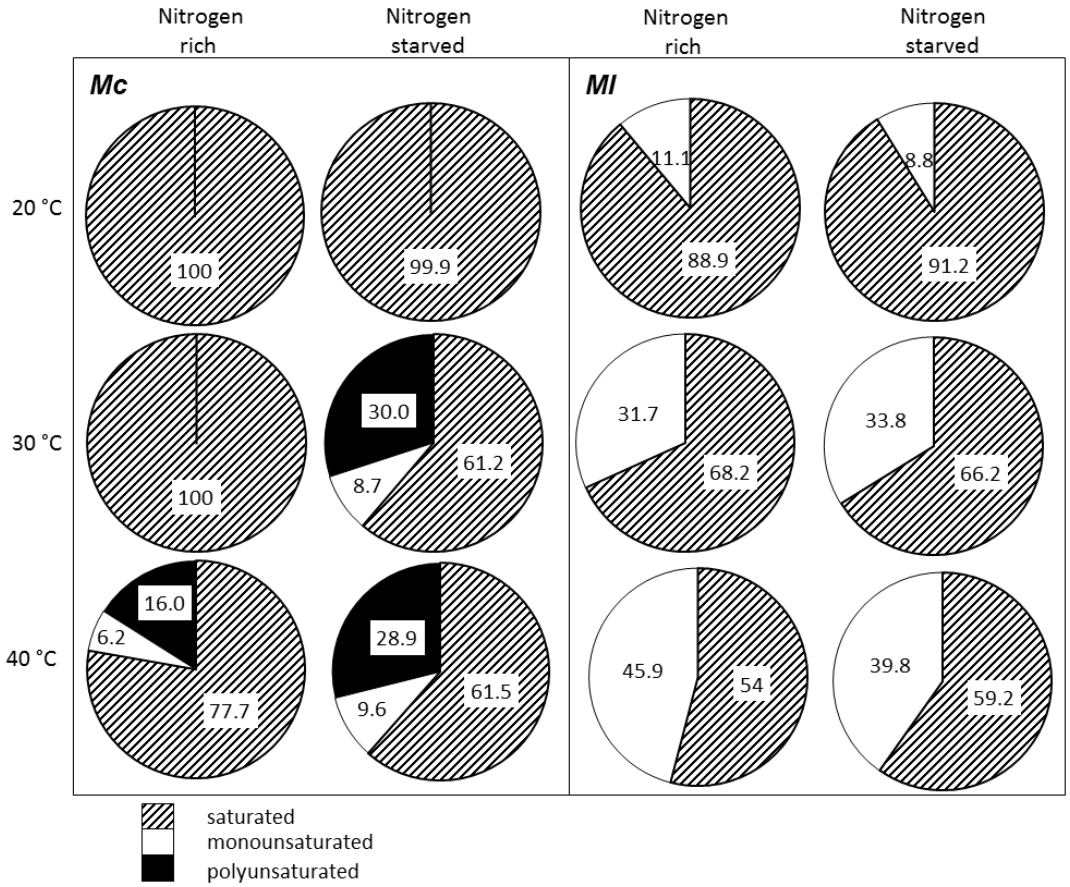


Figure 6.9: FAME profiles of cyanobacterial Roman isolates represented as % total FAMEs.
This data has been summarised into saturates, monounsaturates and polyunsaturates.

6.3 Discussion

Temperature tolerant algae are desirable for reducing culture cooling costs in both open and closed systems using natural sunlight or flue gas as a feed (Chapter 1.3.2). Three temperatures were investigated; low (20°C) medium (30°C) and high (40°C), alongside the effects of nitrogen starvation. Products were assessed by staining with nile red and BODIPY, viewing under TEM and by the extraction and transesterification of lipids into FAMEs, analysed using GC-MS. The experimental design maximised the number of samples for comparison yet spatial constraints did not allow for replicates. This will bestow unknown error margins to the results obtained. This Chapter demonstrated the variability in growth rates and FAME yield and profile of microalgae and cyanobacteria isolated from a single environment. This area of the project also clearly demonstrated the need to develop faster extraction methods for rapid analysis of experimental samples for FAME content and profile.

6.3.1 Thermotolerance of Roman isolates

Cyanobacteria are often found in warm, low nutrient environments (de Winder *et al.*, 1990), and are typically more thermotolerant than eukaryotes (Barsanti and Gualtieri, 2006). This presumably accounts for the ability of Roman cyanobacterial isolates to withstand warmer temperatures, whilst still retaining their productivity. The comparatively poor growth of the eukaryotic isolates compared to the cyanobacteria, suggests that they were present in the baths as debris from other locations and not actively growing *in situ*. *Klebsormidium* spp. is also a common alga found growing in urban areas (Rindi *et al.*, 2007).

In order for the analyses on the effects of temperature to be comparable, all other conditions (light, stirring) were *moderate* in order to be consistent for all species tested. Growth is therefore highly likely to be suboptimal (in some part) for all the species examined. For example, *O. sancta* showed slow growth at 40°C yet is one of the dominant microalgal species in the baths, forming the filamentous 'balloons' around evolved gas bubbles, in both environments it is brown in colour rather than the typical blue-green. Previous studies have found that high light or high temperature conditions change *Oscillatoria* spp. from blue-green to brown and a reduction in its overall productivity is observed (Gribovskaya 2007). So while the results of this investigation are comparable, all species isolated would need to be further investigated to find their optimal growth conditions.

Both *H.* sp. and *C. thermalis* accumulated very low levels of biomass. *Choococcidiopsis* spp. are found in nature in a desiccated state most of the time (Billi *et al.*, 2000). Its resistance to desiccation is likely conferred by its thick cell walls and its tendency to grow in aggregates but it also has very efficient DNA damage repair mechanisms (Billi *et al.*, 2000). The ability to make extra-cellular polysaccharides and spores is common in organisms that can survive these conditions (Billi *et al.*, 2000). With this in mind, it may be likely that *C. thermalis* is not capable of rapid growth, yet it is capable of existing in aggregates which suggests it can grow to high cell densities (data not shown). It may also be likely that whilst under supportive growth conditions the samples simply did not have enough time to adjust.

There is evidence to suggest that there is an increase in productivity of communities as opposed to a monoculture. In addition communities are less prone to invasion of local species, which is often a problem when operating low cost algal ponds (Smith, 2011). It is likely that in the Roman Baths these communities help the microalgal species present to survive, in not only challenging temperatures but also a nutritionally deficient or stressful environment. Calcium, sodium and chloride levels are much higher than standard media used for culturing these groups of algae, which could cause stress (Barsanti and Gualtieri, 2006). Perhaps these conditions account for the slow growth of microorganisms in the baths, which only require draining and cleaning once or twice a year (Byrne, 2009). In many aquatic environments iron is a limiting nutrient for productivity, yet in the baths it is at a similar concentration to most growth media. Silicon is also plentiful in the baths, which accounts for *H.* sp. being able to thrive (Kellaway, 1991).

In a given environment algae may behave very differently. The ‘shock’ of moving an extremophile from its habitat to more favourable conditions (such as culture media) can reduce the vigour of an isolate and make it less productive. Isolates may even take months to recover or adapt to new conditions (Andersen, 2005). This may account for the low productivities observed in the Roman isolates (in particular *H. sp* and *C. thermalis*).

6.3.2 Products of Roman algae

Thick and ridged cell walls can pose as a barrier to nile red stains, rendering it ineffective as a screening method for thick walled algae (Chen *et al.*, 2011). Both Nile red and BODIPY showed varying permeability into cells of different species in particular the cyanobacterial isolates; *C. thermalis, M. chthonoplastes, M. laminosus* and *O. sancta* and as such, staining

was not suitable for the detection of the presence of lipids and/or PHAs. Strong autofluorescence (of cells in all strains) in the same range as nile red emission wavelengths, made the identification of any storage inclusions inside cyanobacterial cells very subjective. TEM was helpful in visualising the cell contents but was not a rapid screening method for microalgal products.

Reducing the nitrogen available to microalgae generally encourages accumulation of lipids. Green algae in particular typically accumulate lipids in the cytosol when under stress (Karatay and Dönmez, 2011). This was not found to be the case for every isolate and lipid accumulation was also dependent on the temperature of the culture. Another factor in the production of biodiesel is the reduction in the growth rate observed under nitrogen limited conditions. For example *C. saipanensis,* though producing marginally more lipids under nitrogen starvation only produced a fraction of the biodiesel as the growth was extremely limited. The cyanobacteria accumulated less of a range of FAMEs than the eukaryotic algae, although still produced high levels of lipid under nitrogen sufficient conditions.

The green algae *C. saipanensis* was found to produce a large amount of polyunsaturated esters under all conditions similar to the reference alga *C. emersonii*. The FAME profile is roughly equivalent to sunflower or soybean oil (Chuck, 2011) and the resulting biodiesel would have similar fuel properties to these biofuels including being more oxidatively unstable. Biodiesel produced from *H.* sp. and *K.* sp. was generally found to be far more saturated than the other microalgal fuels, this effect increased with temperature. Fuels high in saturates tend to have poor low temperature behaviour and a high viscosity. The biodiesel produced from *H.* sp. and *K.* sp. would be unlikely to be usable in high blend levels due to this profile.

The cyanobacteria *M. chthonoplastes* and *M. laminosus* were found to also be extremely rich in saturated esters. An increase in the saturation of lipids has been observed upon incubation for temperature tolerant strains (El-Sheek *et al.,* 1995; Dinamarca *et al.,* 2011; Galhano *et al.,* 2011). Small localized changes in lipid composition can alter membrane physical behaviour (Thompson, 1996). As such, fatty acids are believed to be involved with acclimation of photosynthetic machinery to increased temperatures, by thermally stabilising microdomains of the thylakoid membranes (Balogi *et al.,* 2005). Other studies suggest that the synthesis of fatty acids *and* proteins is required for the enhancement of the thermal stability of PSII (Nanjo *et al*., 2010). Photosynthesis can rapidly decline in cyanobacteria grown above their optimal

temperatures due to degradation and repair of the PSII reaction centres (Dinamarca *et al.*, 2011). Interestingly, the level of saturated esters isolated from *M. laminosus* was reduced when cultured at higher temperature and by 40°C and the biodiesel produced was almost 50% monounsaturated. Monounsaturated esters on balance have the most promising fuel properties, and biodiesel rich in these esters (such as biodiesel derived from rapeseed or olive oil), can be used at higher blend levels than other types of biodiesel.

6.3.3 Further work

Whilst this investigation was designed to be comparable, all species isolated would benefit from further investigation to find their optimal growth conditions. For example, under low light conditions cyanobacterial growth is favoured, and eukaryotic green algae are highly productive under high light conditions (Sheffer *et al.*, 1997). However, due to space limitation in environmental chambers light intensity was at a moderate intensity, so as not to stress cyanobacterial isolates). Small alterations to the media or light level could also greatly improve the growth of some of these isolates. Although some species may not be as productive as others, they may be more suitable for a particular application or set of conditions (e.g. assessing the ability for strains to grow in wastewater). A recently isolated thermotolerant microalga (*Desmodesmus* sp.) was described as having high productivity when cultivated in wastewater supplemented with 2% CO_2 (Huang *et al.*, 2012), which not only highlights the importance of bioprospecting and strain choice but also the value in testing a variety of culture conditions for optimal 'performance'.

Although some species may not be as productive as others, they may well be more suitable for a particular application or set of conditions. Product analysis was only carried out with respect to lipid content and profile, yet microalgae are capable of synthesising a variety of profitable compounds. Primers could be used to confirm the presence of PHA synthases in some of my isolates. TEM images of *M. chthonoplastes, O.santa* and *M. laminosus*, did appear to show some small opalescent granules within the photosynthetic machinery. Finding optimal growth conditions for *H.* sp. may also be of particular interest as diatoms typically have high doubling rates and oil contents. Diatoms are also responsible for 20-25% of total terrestrial primary production and 40% of marine biomass (annually). Like many algae they also produce useful products for food, fuel, pharma and some are also capable of nitrogen fixation (Bozarth *et al.*, 2009).

There is evidence to suggest that there is an increase in productivity of communities as opposed to a monoculture. In an environment such as the Roman Baths 'community living' is often vital. As such the Roman isolates may require the presence of other organisms to exhibit optimal growth. Therefore it may be of interest to see how productivity changes when isolates are grown as a community in nutritionally balanced growth media.

6.3.4 Summary

The aim was to find thermotolerant, oleaginous microalgae that could potentially be used as a source of renewable biodiesel. A number of the species were found to contain high levels of lipids under a range of conditions. Two green algae were isolated, yet both did not exhibit an increased tolerance to elevated temperatures when compared to the eukaryotic control *C. emersonii*. The culture conditions used to screen the microalgae are likely to have been suboptimal for many of the isolates. With optimisation of the growth conditions some of the species screened, they may become effective biodiesel producers. There may also be other advantageous features of the isolates, which have not been examined in this study.

This Chapter highlighted the extreme diversity and variability in growth and FAME yield and profiles of microalgae and cyanobacteria isolated from the same 'extreme' environment (the Roman Baths) and the importance of screening for new species. In essence, it is important to note that the population dynamics of microalgae in their natural environments is complex. The isolates may be capable of being more productive, if their conditions were slightly altered, rather than at a 'conservative' value so all algae could be grown together (due to limited growth equipment). This area of the project also clearly demonstrated the need to develop rapid and efficient extraction methods, as this limits the rate of analysis of FAME content and profile. This is further investigated in Chapter 7.

7. INVESTIGATION OF METHODS FOR EFFICIENT EXTRACTION OF ALGAL LIPIDS

7.1 Introduction

7.1.1 Methods of extraction

Fatty acid composition is vital to determining the quality and physical properties of biodiesel. It is therefore beneficial to have a rapid FAME extraction and screening for algae from environmental samples (Guzman *et al.,* 2010). Accurate lipid profiling of algae is usually carried out using GC-MS, which not only requires a large sample of biomass but is also coupled with lengthy sample preparation often involving lyophilisation, extraction, purification and transesterification (Mutanda *et al.,* 2011). Comparisons of different lipid extraction methods on different algal species have been reported, in attempts to shorten and simplify the extraction of FAMEs to more rapidly obtain experimental results.

The extraction of FAMEs is usually a 2-step process. Firstly the extraction of the TAGs from biomass, followed by a transesterification reaction yielding FAMEs (and glycerol as a by-product) commonly analysed by GC-MS (MacDougall *et al.*, 2011) yet can also be analysed with FT-IR spectroscopy (Pistorius *et al.,* 2009). After transesterification water is added to remove methanol and other impurities (Cooney *et al.*, 2009).

There are many ways to extract lipids from microalgae (Figure 7.1), pressing, homogenization, milling, solvent extraction, supercritical fluid, enzymes, ultrasonic-assisted extraction, osmotic shock and novel methods such as ‘milking’ (Packer 2009). Depending on moisture content, different processing techniques may be required. For example dry biomass is suitable for gasification, pyrolysis or combustion, whereas wet material is more suited to enzymatic fermenting (McKendry, 2002).

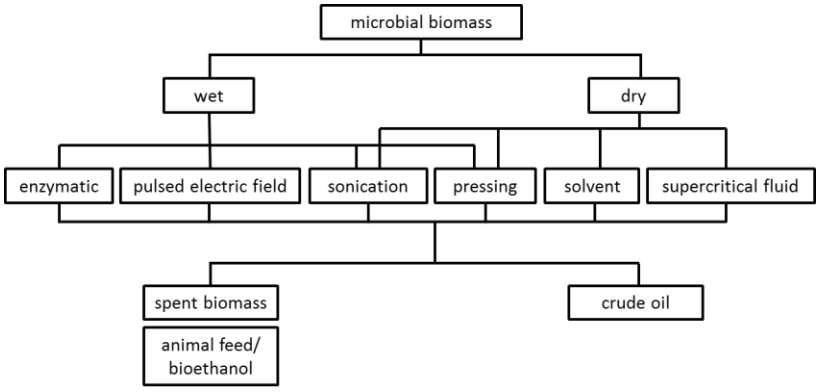


Figure 7.1: A generalised flow diagram showing the variety of extraction methods which can be applied to wet and dry algal biomass.
Adapted from Mercer and Armenta (2011).

All extraction methods have their benefits and drawbacks and may be suitable for certain applications only or used in conjunction with one another (Table 7.1). Physical and chemical methods usually involve the addition of large volumes of solvent (Mercer and Armenta, 2011). Water associated with biomass can block solvent from getting in and out of cells (Cooney *et al.,* 2009). Drying of material can reduce the quantity of solvents needed (Hass and Wagner, 2011), yet requires a higher energy input (dewatering algal biomass) and is undesirable. Solvent must be able to permeate biomass fully and match polarity of the product to be extracted. Benzene, cyclohexane, hexane, acetone and chloroform have proven effective on microalgal pastes (Mercer and Armenta, 2011). A mixture of two or more solvents can drastically alter the extraction properties of a solvent. Co-polar solvents can make membranes more porous to better extract lipid from inside cells (Cooney *et al.,* 2009). Chloroform:methanol (2:1) as a solvent routinely yields the highest FAME content after transesterification (Johnson and Wen, 2009). Solvent extraction alone (i.e. in a soxhlet) is rarely sufficient to extract all oil (Mercer and Armenta, 2011). Increased temperature can increase solvation power of a solvent (by overcoming solute-solute and solute-matrix interactions). Additionally, increased pressure helps transport solvent to products trapped in pores and matrices. Mechanical methods can offset the need for higher temperature and pressure (Cooney *et al.,* 2009).

Table 7.1: Advantages and disadvantages of a variety of lipid extraction methods.
Adapted from Mercer and Armenta (2011).

Extraction Method	Advantages	Disadvantages
Solvent extraction	Solvents are relatively inexpensive results are reproducible	Most organic solvents are flammable and toxic, solvent recovery is expensive, large volumes required
Pressing	Easy to use, no solvent needed	Large amount of sample required, slow process
Sonication	Reduced extraction time, reduced solvent use, improved release of cell contents	High power consumption, generation of radicals, difficult to scale up.
Beadbeating		
Supercritical fluid	Non-toxic, so solvent residue in product, non-flammable, simple operation	High power consumption, difficult to scale up.
Microwave	Rapid extraction, high yields	Needs optimisation, difficult to scale up, may affect FAMEs
Enzymes	Could be efficient, safe	Expensive

Enzymes have the potential to partially or fully degrade the cell wall, yet are reliant on the nature of the species in use and often too expensive to use on an industrial scale. Enzymes for transesterification whilst expensive show good tolerance to free fatty acid chain levels. As such, immobilization of enzymes may prove useful in process integration (Um and Kim, 2009). Ultra-sonication at high frequencies causes 'cavitation' (creating bubbles in solvent which violently collapse) producing shockwaves which can disrupt the cell wall. However this is an energy intensive process, which also heats the solvent very quickly and is difficult to scale up (Mercer and Armenta, 2011).

Supercritical fluid extraction is a rapid method of extraction, eliminating the use of solvents and as such, gives a clean product without the need for purification (no heavy metals present, free of inorganic salts). Alterations to its specificity can be made with the addition of solvents, pressure, temperature, CO_2 flow rate and extraction time. Yields are high, yet the process is expensive and algae must be dry as moisture acts as barrier, reducing contact algae and CO_2 (Mercer and Armenta, 2011).

Microwaving is a rapid extraction method that works on principle of selective heating. Microwaves directly affect polar materials and when used on biomass, trace quantities of moisture evaporate generating a significant amount of pressure that stresses the cell to rupture and release contents (Mercer and Armenta, 2011). However the process may be difficult to scale up and still requires optimisation. Microwaves may also produce oxidative agents which could damage algal products. Preliminary results seem to suggest a huge reduction in extraction time and sufficiently more oil recovered (Balasubramanian *et al.*, 2011).

Knowledge of intact lipid profiles could be useful such as selection of a strain for further research (e.g. by UHPLC-MS, currently under development at Bath). *In situ* transesterification has been shown to improve oil extraction (Hass and Wagner, 2011).Transesterification requires the addition of alcohol (acts as a catalyst) and heat, performed at a high pH. Unsaturated fatty acids can be problematic as they can create cross links (Singh *et al.,* 2011).

Extraction followed by transesterification often gives incomplete recovery of lipids (Cooney *et al.,* 2009). Direct transesterification of algal biomass has been proven to be very effective (at least 15-20% over extraction-transesterification reactions) (Mercer and Armenta, 2011); in particular for use with microwave extraction (Balasubramanian *et al.,* 2011 and Patil *et al.,* 2012). With this process it is important to include a cooling system (to keep temperatures <100°C) for the solvent and uniform mixing (Patil *et al.,* 2010). Small changes in temperature (5°C) and extraction time (min) can make a large difference in the quantity of recovered FAMEs (Balasubramanian *et al.,* 2011).

Species choice has a big impact on the method of extraction. For example oleaginaceous *Botryococcus braunii*, is capable of growing in the presence of the biocompatible solvent tetradecane in a biphasic aqueous-organic bioreactor. As the alga generates lipids and is mixed in culture, oils are drawn into the tetradecane (process known as 'milking'), eliminating the need to disrupt the cell wall (Zhang *et al.,* 2011). *Botryococcus* spp. is an algaenan-producing species (Chapter 3.1.3) and the production of algaenan in *Botryococcus* spp. is believed to be linked to synthesis and exportation of oleic acids to the cell wall (Templier *et al.,* 1993). Theoretically, other algaenan-producing species (such as *C. emersonii*) may also be suitable for culture in a biphasic 'milking' PBR.

Efficacy of the extraction method varies enormously depending on species and exact conditions deployed. Cell shape and structure of different species, no matter how minor can have an effect (Mercer and Armenta, 2011). For example a study by Lee *et al.* (2010), compared *C. vulgaris*, *B.brauni*, and *Scenedesmus* sp. over a variety of extraction methods; soxhlet, autoclaving, bead beater, microwave, sonication and osmotic shock. Microwaving was consistently most successful in this study for maximum oil extraction, however other methods varied in their efficacy in extracting oils depending on species (Figure 7.2). Similar results were found in a study by Prabakaran and Ravindran (2011).

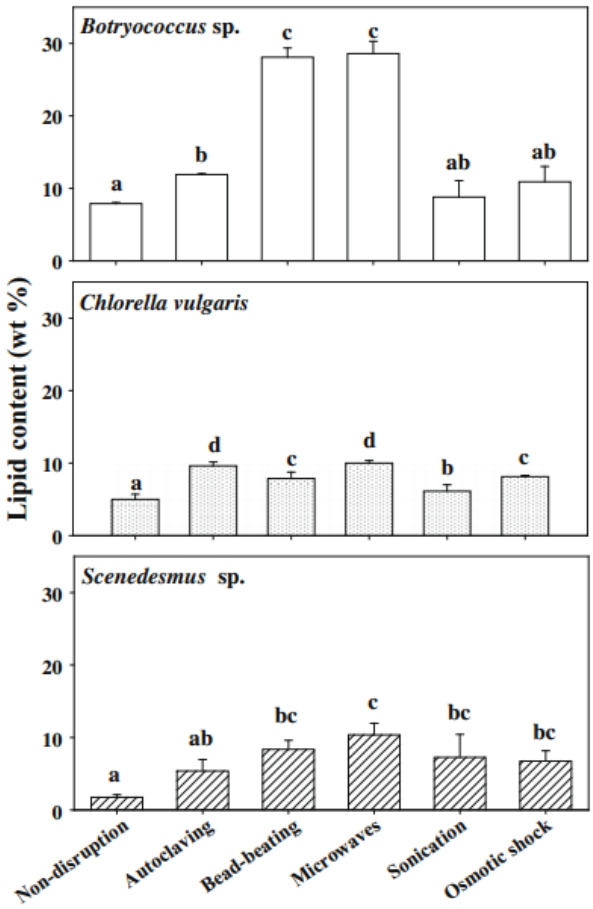


Figure 7.2: A comparison of different lipid extraction methods using *Botryococcus* sp., *C. vulgaris* and *Scenedesmus* sp.
From Lee *et al.* (2010).

The extraction methods described are also commonly used for extraction of other products (such as β-carotene) (Hejazi *et al.*, 2004). It is important to note that extraction methods used in the laboratory for recovery of microalgal oils, may not apply to industrial scale systems and certain methods of extraction will be more or less efficient depending on the details of operation (Mercer and Armenta, 2011). Upon scaling up an extraction process, the vast quantities of solvent required become impractical. Residual solvent contamination not only affects the final product but also machinery parts. Inorganic salts within solvents can reduce selectivity (i.e. additional polar extracts can result in poor flow characteristics). Recovery and recycling of solvents also adds to the expense (Cooney *et al.*, 2009).

7.2 Results

7.2.1 Preliminary extraction experiment

Solvent soxhlet extraction is the common method used for lipid extraction from algae, yet is very time consuming, increasing the time required to analyse products from various experiments. As such it was of interest to examine other, faster methods of extraction. As FAME profile is important in assessing the quality of biofuel, extraction methods used must not affect the FAME profile. An 'old' (lag phase) lyophilised culture of *C. emersonii* was initially subjected to three extraction methods in order to determine which was most efficient at extracting FAMEs and if the FAME profile was affected by the method of extraction. Compared extraction methods included soxhlet, sonication and extraction in a domestic microwave. No repeats were carried out due to limited biomass production capacity at the time.

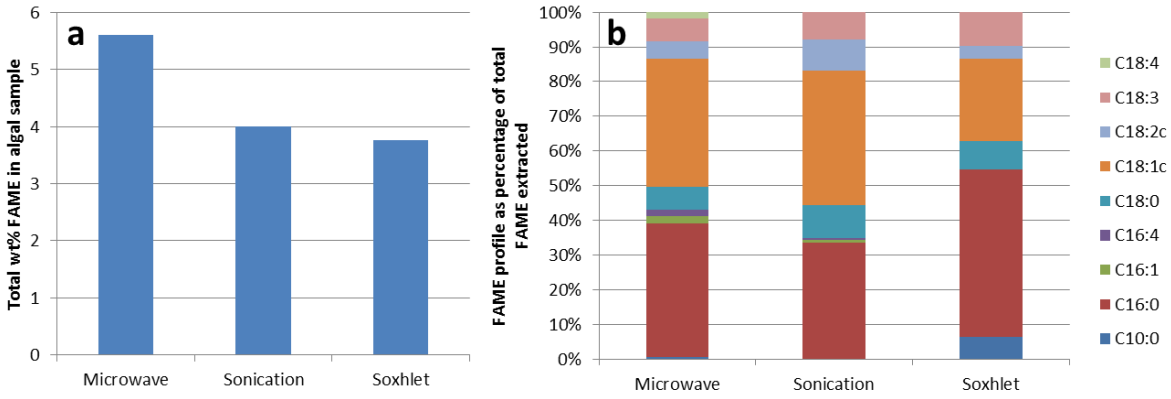


Figure 7.3: FAME content and FAME profile of lipids extracted from a lag phase culture of *C. emersonii* using three different extraction methods.
(a) FAME content (as wt% total algae) and (b) FAME profile (as a percentage of total FAMEs) having undergone different extraction methods; microwave, sonication and soxhlet. No repeats were performed due to limited biomass.

Microwave extraction was the most efficient method of extracting lipids in terms of total quantity extracted and processing time (Figure 7.3). FAME content was largely unaffected between the different extraction methods with the exception of soxhlet extraction, where less $C_{18:1c}$ and more $C_{16:0}$ is extracted. This difference may have been accounted for had repeats been carried out. As soxhlet extraction is time consuming (and preliminary results show microwaving could become a preferable method), the experiments were repeated and replicated with the addition of integrated transesterification, beadbeating and an automated microwave reactor (Anton Paar MAS24 Monowave 300).

7.2.2 A comparison of extraction methods

A log phase culture of *C. emersonii* was lyophilised and subjected to different extraction methods (with 3 replicates for each method). Sonication, beadbeating and microwave '1' and '2' were carried out in water with subsequent 'Bligh and Dyer' extraction, whereas the other methods used solvent ($CHCl_3$: MeOH 2:1). Anton Paar '2' and microwave '2' both had the addition of H_2SO_4 in an attempt to directly transesterify lipids during extraction.

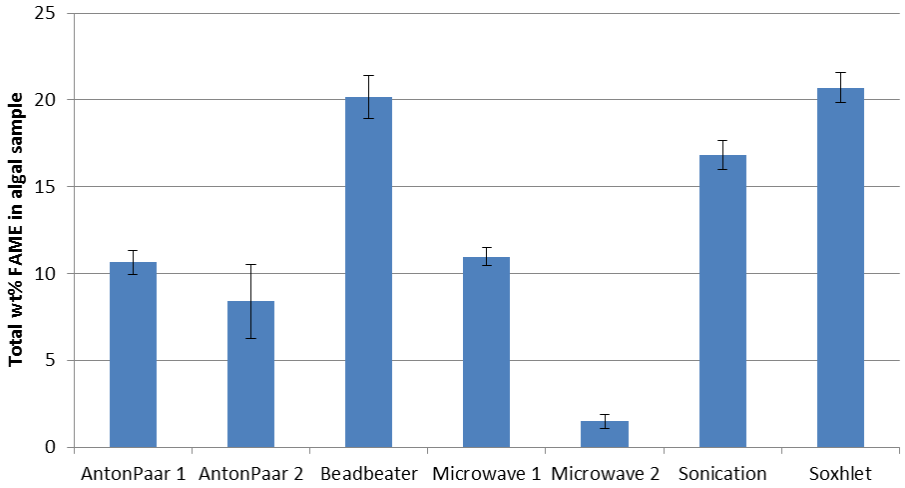


Figure 7.4: Total FAMEs (as wt% of algal biomass) extracted from log phase culture of *C. emersonii* using different extraction methods.
Three replicates were performed for each experiment, error bars = S.D.

Beadbeating and soxhlet proved most efficient at extracting FAMEs (Figure 7.4). This is contrary to the preliminary experiment (in which microwaving extracted the highest quantity of FAMEs) which could be a result of error and/or the quality of algal biomass (fresh log phase culture rather than 'old' stationary phase culture). Although soxhlet proved to be most efficient at extracting lipids from biomass in (Figure 7.4) it is still a lengthy process (requiring up to 3 days to process), with sonication and beadbeating a significantly faster extraction method (requiring up to 1 day to process). Microwave and AntonPaar extraction yielded very low levels of FAMEs (half the quantity of other extraction methods). There were no major differences in FAME profile between the different extraction methods (Figure 7.5).

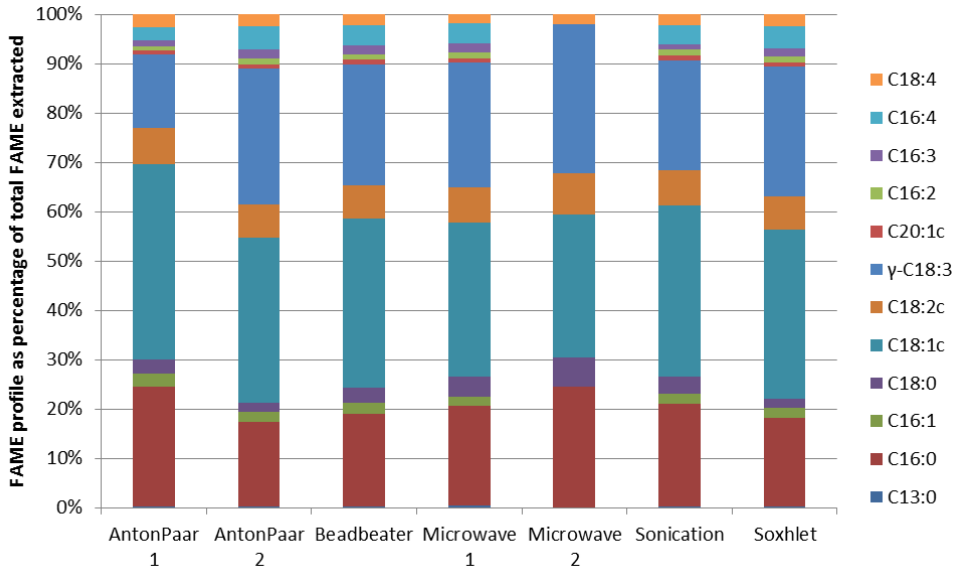


Figure 7.5: FAME profiles (as a percentage of total FAMEs), from a log phase culture of *C. emersonii* having undergone different extraction methods.

The difference in quantity of FAMEs extracted for the microwave methods was investigated further by analysing samples by NMR for any degradation products. Acids were found in all microwave and AntonPaar samples, which when totalled with FAMEs detected with the GC-MS gave a similar value to soxhlet extraction for total FAMEs. This suggests that approximately half of the extracted FAMEs using microwave methods, underwent degradation to acids. FAME profile however was unaffected, which means all FAMEs are equally sensitive to thermal degradation. Due to the speed of microwave extraction, once optimised it would be very preferable to soxhlet extraction. It is also important to note that different extraction methods may be better at FAME extraction depending on choice of species. NMR spectra for Soxhlet and 'AntonPaar 2' are given in full alongside GC-MS data in Appendix G.

7.2.3 Microwave extraction investigation

The AntonPaar MAS24 Monowave 300 underwent further experimentation in an attempt to optimise the extraction of lipids, without the breakdown of FAMEs. Many studies investigating microwave extraction have a form of internal cooling, to cool the reaction vessel (Patil *et al.*, 2010). The AntonPaar microwave did not have this option. Balasubramanian *et al* (2011) found that temperatures between 80°C and 100°C are sufficient for microwave extraction of microalgal lipids. It was decided to investigate the effect of reaction time with temperature, examining one low value of 80°C and a high value of 120°C.

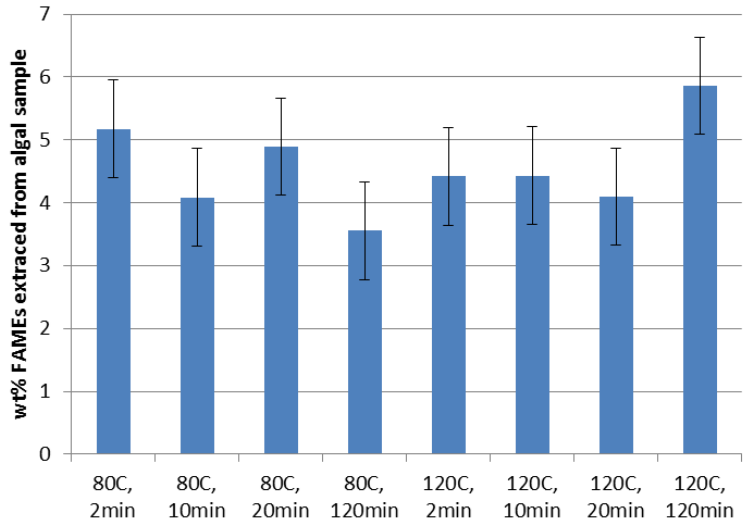


Figure 7.6: Total FAMEs extracted (as wt% algal biomass) using the AntonPaar scientific microwave at different temperatures and exposure times.
Three replicates were performed for each experiment, error bars = S.D.

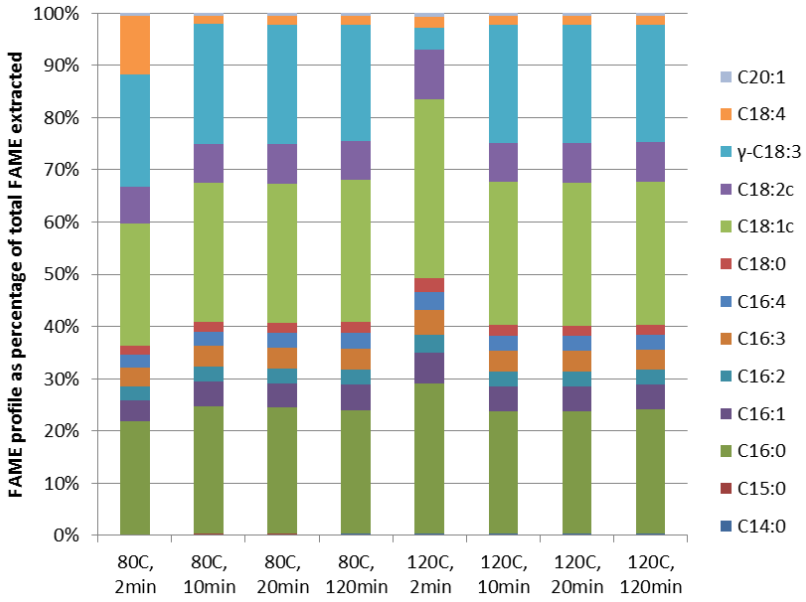


Figure 7.7: FAME profiles (as percentage of total FAMEs) using the AntonPaar scientific microwave at different temperatures and exposure times.

Of all conditions tested for the AntonPaar scientific microwave, none showed full extraction of FAMEs (content estimated at ~21wt%, Figure 7.4) without being coupled with degradation (Figure 7.6). Degradation of FAMEs was approximately equal between samples and between types of FAME (Figure 7.7). The method still has potential for further investigation and possible optimisation, yet unfortunately due to time constraints was not able to undergo further method development.

7.3 Discussion

Fatty acid composition is crucial to determining the quality and physical properties of biodiesel (Guzman *et al.*, 2010). As such a rapid FAME extraction protocol for faster analysis of experimental data is highly desirable. This Chapter investigated the effect of extraction method on total quantity of FAMEs extracted and their profile, with the intention of accelerating sample analysis. Extraction methods investigated included soxhlet solvent extraction, beadbeating, sonication and microwave assisted extraction (using both a domestic microwave and a scientific microwave reactor (AntonPaar MAS 24 Monowave 300)). Both microwave methods were also investigated for the direct transesterification of lipids during extraction. Soxhlet was capable of extracting the highest quantity of FAMEs yet required up to 3 days to process. Beadbeating was a much more rapid method by which to extract high quantities of FAMEs (requiring up to 1 day of processing). It was found that microwave extraction was capable of rapidly extracting all lipids, yet required optimisation to prevent degradation of FAMEs. The quality of the biomass used was found to have an effect on the efficacy of extraction methods tested.

7.3.1 Comparison of extraction methods

The biomass used for the preliminary extraction method experiment not only had low lipid content, yet in addition may have started to degrade. If error values from the second set of experiments could be applied to the preliminary experiment, all methods could be said to have yielded a similar FAME quantity. The age and degradation of biomass could account for there being no discernible differences between extraction methods in the preliminary experiment.

Using GC-MS data alone, soxhlet extraction followed by beadbeating and sonication were the most effective at extracting microalgal lipids. Beadbeating and sonication was significantly more rapid than soxhlet extraction (requiring 3-48hrs to extract as much lipid as possible). Microwave extraction proved effective at extracting all lipids but showed breakdown of FAMEs to acids (detected by NMR) which accounted for the reduction in FAMEs seen in the GC-MS.

It is important to note that with all extraction methods the resultant ‘algal lipid extract’ is not a ‘clean’ extract consisting solely of TAGs, there are many other contaminants most notably chlorophyll. Other extraction methods such as supercritical CO_2 are much more specific.

7.3.2 Microwave extraction using the AntonPaar MAS24 Monowave 300

As the facilities at Bath did not yet allow the production of large quantities of biomass (and that which was produced was in high demand from other project groups) only a small number of experiments were carried out to attempt to optimise extraction using the scientific microwave. Both temperatures investigated (80°C and 120°C) resulted in thermal degradation of FAMEs to acids. The tight seal of reaction vessels and the subsequent increases in pressure compared to other methods, may mean that significantly lower temperatures can be used. The absence of an integrated cooling system may also have had a detrimental effect (present in literature claiming high levels of lipid extraction using microwaves). Further preliminary experiments by Sargeant (2011) in the Department of Chemistry (University of Bath) found that cycling of temperatures can cause pressure spikes, which may aid in extraction of lipids from microalgae.

7.3.3 Further work

Further method development for the optimisation of direct transesterification and extraction of algal FAMEs using the AntonPaar MAS24 Monowave 300 would be very meaningful, as this extraction method is significantly faster than any other, requiring fewer steps. Lowering temperatures to 70°C or 60°C with temperature cycling (to investigate the effect of pressure 'spiking' on cell lysis) would be invaluable, in particular if it could reduce the degradation of FAMEs to acids. It would be beneficial to see how an optimised method using the scientific microwave would compare across different algal species.

7.3.4 Summary

Extraction of microalgal lipids and transesterification is a lengthy process which requires streamlining and optimisation for the rapid screening of experimental samples. Microwave assisted extraction and direct transesterification holds promise, yet requires further investigation for optimisation. It is likely that efficacy of extraction methods may change depending on the microalgal species.

8. GENERAL DISCUSSION

8.1 The enigmatic microalgal cell wall

One of the initial aims of this project was to lower the energy required for product extraction. If algal cells could undergo controlled lysis in their culture medium (either through autolysis or with the addition of enzymes) this would remove the need for energy intensive dewatering and/or physical methods of cell disruption, allowing lipids to float to the surface for removal.

The majority of literature examining structure and composition of the cell wall in common microalgae (i.e. specific strains of *Chlorella* sp.) were published in the 1980-1990s (Pienkos and Darzins, 2009), with more recent literature focusing on scientifically or economically valuable species such as *Chlamydomonas* spp. (Liu *et al.,* 2006) and *Haematococcus* spp. (Hagen *et al*., 2002). These papers usually give an insight into the overall physical structure of the cell wall (using imaging techniques) (Hagen *et al*., 2002), yet chemical analyses are simple, rarely extending beyond sugar composition (Burczyk *et al.,* 1995). Additionally microalgae are known for altering their cell wall composition in response to their environment (Cheng *et al.,* 2007).

The experimental investigations presented in this thesis, found the algal cell wall is highly variable in morphology and strength between different species. Staining was ineffective for cell wall studies with the majority of our species, due to the presence of the biopolymer 'algaenan', which was later found to confer enzymatic resistance to algal cells. Despite the failure to develop an enzymatic-method to lyse algal cells or ascertain cell wall composition, a replicable technique for measuring cell wall strength (using sonication) revealed significant sensitivity to changes in the cell wall. However, TEM gave valuable insight into the variety of conformations of algaenan and other cellular components. Using TEM images and sonication results, algaenan was found to provide significant physical strength to cells (as in *P. ellipsoidea,*), with cell strength notably increasing as algaenan content increased or a thick cell wall was present in addition to algaenan as in *C. emersonii*.

Until 2009 the link between poor staining of cells and presence of algaenan had not been reported. Previous literature had revealed the presence of a chemically and physically resilient

biopolymer, primarily referred to as sporopollenin. Published data by Zych *et al.* (2009) not only made this link, but also matched staining observations for our strains.

Despite little information on its structure, algaenan is believed to be chemically similar to cutin, suberin and plant sporopollenins (Kontkanen *et al.,* 2009). The study of fungi capable of digesting these polymers (Zimmermann and Eeemüller, 1984), may therefore provide enzymes capable of algaenan digestion. Preliminary investigations examining the effect of fungal enzyme mixtures on algaenan digestion have been described and despite no evidence suggesting digestion of algaenan (extracted from *C. emersonii*) by *Fusarium oxysporum* f.sp. *elaeidis*, the experiments did highlight areas for method development in the analysis of algaenan digestion using live fungi.

Algaenan may not only provide a barrier to efficient product extraction but could prove problematic for dewatering using flocculants. Those with algaenan in the outer wall will be less affected by flocculants tailored toward cell wall sugar composition (Cheng *et al.,* 2007). Alterations to the cell wall of microalgae can greatly affect the efficacy of flocculants (Cheng *et al.,* 2007). Developing an understanding of the behaviour of the dynamic microalgal cell wall is not straightforward, yet remains valuable in the development of algal biotechnology.

8.2 Improving microalgae by mutagenesis

Genetic modification is commonly used to generate interesting strains for study and is often used in the improvement of strains for biotechnology (Campbell and Reece, 2002). Genetic tools for use in the manipulation of the microalgal genome are currently undergoing development and the genomes for only a few species are publicly available online (Walker *et al.,* 2005). The genus *Chlorella* spp. (a common genus for study) contains many oleaginaceous microalgae (Karatay and Dönmez, 2011), yet no widely applicable genetic tools have been described in literature (data not shown). Due to this, mutagenesis of *C. emersonii* presented in this thesis aimed to generate interesting and useful phenotypes and develop a rapid screening method for the isolation of cell wall mutants for study.

The use of UV to generate mutants of *C. emersonii* required a high-throughput screening method for the detection of cell wall mutants. This presented a challenge due to the unusual staining behaviour exhibited by algae containing algaenan, which includes *C. emersonii*. Algaenan provides a barrier to stains, preventing their penetration to the inner wall, rendering

common cell wall stains ineffective for visualisation of the cell wall. However, this was used to an advantage as a screening tool for the selection of algaenan-free mutants using FACS as a high-throughput method for the sorting of cells. Unfortunately no viable cell wall mutants were obtained using these methods. One potential explanation is that multiple rounds of selection by FACS may have been required before a phenotypically distinct population emerged due to a high number of false positives. However UV mutagenesis did yield a pale mutant of *C. emersonii,* with different growth characteristics and an altered metabolism compared to the control. Regrettably there was not enough time during the end phase of this project to allow for multiple rounds of selection.

Molecular tools for genomic manipulation of microalgae for product yield enhancement and metabolic control are currently undergoing rapid developments in this area (Day *et al.*, 2012). There is evidence to suggest that genetic tools will need to be tailored to each genus or even species of algae (Spicer, 2012). From this it is reasonable to expect that in the near future much literature will be published describing advances in the field of algal genetics.

8.3 The ideal biodiesel alga – the search continues

An important consideration for any commercial algal culture facility is the species choice. Not only must it have a high lipid content but also suitable growth requirements and good growth kinetics (Ratha and Prasanna, 2012). A process may be limited to specific requirements for culture conditions such as waste water, or final end product for example human food grade supplements.

The capacity for microalgae to assimilate CO_2 and other waste materials from which to synthesise an assortment of compounds, coupled with their diversity has highlighted the value of bioprospecting unusual natural environments for potentially profitable species suited to aquaculture (Yang *et al.*, 2012). Thermophilic species of microalgae are desirable not only for culture in geographic regions with high incident sunlight but also for aquaculture utilising warm flue gas as a supplementary CO_2 feed (Huang *et al.*, 2012 and Ebery and Ely, 2012).

Thermophilic strains of microalgae have largely been described from the US (Aquatic Species Program) and Japan due to significant investments during the 1980-1990s and several thermophilic springs in those regions (Ratha and Prasanna, 2012). Few thermophilic strains have been described from any other geographic location. The Roman Baths is the hottest

spring in the UK (Byrne, 2009), but has only been previously subjected to chemical evaluation and little other scientific study (Kellaway, 1991).

Bioprospecting of the Roman Baths revealed a great diversity of microalgal strains with a range of morphologies present in the thermal waters. Temperature tolerance experiments on these isolated microalgae also revealed changes in fatty acid profile with changing temperature and nitrate conditions. Regrettably there was insufficient time during the project to fully optimise culture conditions to determine the full potential of the isolated species, which would benefit from further studies. For example, *Hantzschia* sp. and *C. thermalis* grew poorly and slowly, yet *Hantzschia* spp. has been described as an oil rich species (Ratha and Prasanna, 2012) and *C. thermalis* was observed to achieve a remarkable cell density (data not shown). Four filamentous strains of microalgae were also isolated from the Roman Baths which could significantly reduce the energetic cost of dewatering (Uduman *et al.,* 2010). One of the filamentous cyanobacterial isolates also contained heterocysts (that fix atmospheric nitrogen) which could lower nitrate requirements (Greenwell *et al.*, 2009). A recently isolated thermotolerant microalga (*Desmodesmus* sp.) was described as having high productivity when cultivated in wastewater supplemented with 2% CO_2 (Huang *et al.*, 2012), which not only highlights the importance of bioprospecting and strain choice but also the value in testing a variety of culture conditions for optimal 'performance'.

Bioprospecting demonstrates the inherent variability in microalgal physiology, chemistry and behaviour and how this is subject to change depending on culture conditions. Technical challenges in the field of algal genetic manipulation and its use in biotechnology suggests that bioprospecting for algae with unique properties will remain invaluable. All the isolated roman strains were deposited to the Culture Collection of Algae and Protozoa (CCAP, Oban, Scotland) and are publicly available (Smith-Bädorf *et al.*, 2012 (submitted for review))

Due to the restraints on the use of GM organisms in industry and the challenges posed by microalgal genetic manipulation, bioprospecting and the study of naturally (perhaps co-occurring) organisms may become key in solving problems with algal aquaculture. For example, nitrogen-fixing cyanobacteria or co-culturing with nitrate-secreting bacteria could lower nutrient costs, or co-culturing with bacteria or fungi may be beneficial for the flocculation of microalgae (Zhang and Hu, 2012). The isolation and study of useful enzymes from organisms which digest algae could also become valuable in studying the cell wall or reducing the cost of harvesting.

I believe the most valuable aspect of all the work presented is having developed a broad variety of techniques (culturing, measuring growth, mutagenesis, bioprospecting, molecular identification, cell wall imaging, product extraction and analysis) for studying algae, which prior to this project had not existed at the University of Bath. The multidisciplinary nature of this project across the department of Chemistry and Chemical Engineering at the University of Bath and the University of the West of England, greatly enriched the possibilities open to this project.

8.4 Personal reflections

Climate change and 'peak oil' is a symptom of unsustainable energy use. 'Modern' economically developed societies are built on constructs which require continuous growth. The natural physical world cannot support continuous growth, but undergoes cycles of energy conservation (fasting), regeneration (growing), diversification (creativity/ exploration) and redistribution of energy. As such, energy solutions should be diverse requiring collaboration from a variety of sectors to encourage smaller 'tailor made' solutions, for example small localised power generation and the implementation of a decentralised 'permeable' energy network and to feed back into an energy grid (Pienkos and Darzins, 2009). In addition, carbon-sequestering technologies to 'lock away carbon' (for example 'hemp-crete'), should also be encouraged.

I believe we have the capacity to exist in the developed world sustainably, with the largest challenge implementing or making the transition to new technologies, with our current 'fossilised' infrastructure. Energy consumption is currently quite wasteful. Behavioural changes and the way energy is viewed could have a big impact on the way we use energy. However, particularly due to the current financial climate, choices on energy generation will be heavily influenced by cost. Choices should still be offered with subsidisation or incentivisation of 'greener' options.

For the majority of new algal technologies initial capital costs are high and will require subsidies. All research into algal biotechnology is very valuable if only for the potential to synthesise products whilst remediating CO_2 and waste from industrial streams, turning waste into profit. As algae are responsible for the fixation of large quantities of atmospheric CO_2 and are the primary producers in aquatic systems, understanding their biology and ecology

will become important as the climate changes. Microalgal biodiesel will still require years of development in order to reach a commercial scale whereby the advantages are seen. With present technology the UK aquaculture is more suited to high value products (Parker, 2012).

The realisation of commercial algal biotechnology is underway, yet there is still a long road ahead (Ratha and Prasanna, 2012). I believe 'industrial symbiosis' of algal technologies (integrating bioremediation and bioproduction), will be the key to success of greener energy generation and should be encouraged by the cohesion of research groups, industry and government (Sivakumar *et al.*, 2012). Costs can be substantially reduced by co-location (Parker, 2012), which would require flexibility in process designs and potentially local laws, to account for unique parameters specific to a single plant.

I strongly believe that the progress of microalgal research is heavily reliant of strong clear communication, pitched at the right level, not only between researchers but also between industry and policy makers (Schlarb-Ridley, 2012). In such a currently competitive field there is much positive hype and highly guarded information, which can greatly hinder progress. Conference talks discussing the benefits of algal biotechnology were plentiful during the project, but largely did not examine the research itself. Without the collaboration of other researchers, this project would have been significantly slowed. The value of communicating research led to the organisation of the 'algae for renewable energy' symposium, which took place at the University of Bath on 12th January 2011, and was continued by UCL on the 13th February 2012 ('algal biotechnology: biofuels and beyond'). The symposium series intends to continue to bring algal researchers together over the next few years and so far has demonstrated to be a valuable and enjoyable experience for all those who attended.

9. REFERENCES

Abed, R.M.M., Köster, J. (2005) The direct role of aerobic heterotrophic bacteria associated with cyanobacteria in the degradation of oil compounds, *International Biodeterioration and Biodegradation*, **55**: 29-37

Afi, L., Metzger, P., Largeau, C., Connan, J., Berkaloff, C., Rousseau, B. (1996) Bacterial degradation of green microalgae: incubation of *Chlorella emersonii* and *Chlorella vulgaris* with *Pseudomonas oleovorans* and *Flavobacterium aquatile*, *Organic Geochemistry*, **25**: 117-130

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. (2004) *Essential cell biology* (2nd ed), Garland Science, Abingdon UK

Ali, S. (2011) *pers.comm.*, 12th Jan 2011, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Allard, B., Templier, J., Largeau, C. (1998) An improved method for the isolation of artifact-free algaenans from microalgae, *Organic Geochemistry*, **28**: 543-548

Allard, B., Templier, J. (2000) Comparison of neutral lipid profile of various trilaminar outer cell wall (TLS)-containing microalgae with emphasis on algaenan occurrence, *Phytochemistry*, **54**: 369-380

Allard, B., Templier, J. (2001) High molecular weight lipids from the trilaminar outer wall (TLS)-containing microalgae *Chlorella emersonii*, *Scenedesmus communis* and *Tetraedron minimum*, *Phytochemistry*, **57**: 459-467

Allard, B., Rager, M-N., Templier, J. (2002) Occurrence of high molecular weight lipids (C_{80+}) in the trilaminar outer cell walls of some freshwater microalgae. A reappraisal of algaenan structure, *Organic Geochemistry*, **33**: 789-801

Andersen, R.A. (2005) *Algal culturing techniques*. Elsevier Academic Press, Burlington USA

Andrews, J.N., Burgess, W.G., Edmunds, W.M., Kay, R.L.F., Lee, D.J. (1982) The thermal springs of Bath, *Nature*, **298**: 339-343

Montelone, B.A. (1998) *Mutation, mutagens and DNA repair* [online]. Available from: http://www-personal.ksu.edu/~bethmont/mutdes.html#mutagens [Accessed 1st May 2012]

Atkinson TC, Davison RM (2002) Is the water still hot? Sustainability and the thermal springs at Bath, England. *Geological Society London, Special Publications* **198**:15-40

Bajaj, Y.P.S. (1990) *Biotechnology in agricultrure and forestry 13: wheat*, Springer-Verlag, Heidelberg Germany

Balasubramanian, S., Allen, J.D., Kanitkar, A., Boldor, D. (2011) Oil extraction from *Scenedesmus obliquus* using a continuous microwave system - design, optimization and quality characterization, *Bioresource Technology*, **102**: 3396-3403

Balogi, Z, Torok, Z., Balogh, G., Josvay, K., Shigapova, N., Vierling, E., Vigh, L., Horvath, L. (2005) Heat shock lipid in cyanobacteria during heat/light-acclimation, *Archives of Biochemistry and Biophysics*, **436** : 346-354

Barbosa, M.J., Hadiyanto, Wijffels, R. (2003) Overcoming shear stress of microalgae cultures in sparged photobioreactors, *Wiley Periodicals*, doi: 10.1002/bit.10862

Barsanti, L., Gualtieri, P. (2006) *Algae anatomy biochemistry and biotechnology*. Taylor & Francis Group, London UK

Bernard, O. (2011) Hurdles and challenges for modeling and control of microalgae for CO_2 mitigation and biofuel production, *Journal of Process Control*, **21**: 1378-1389

Bernards, M.A. (2002) Demystifying suberin, *Botany*, **80**: 227-240

Billi, D., Friedmann, E.I., Hofer, K.G., Caiola, M.G., Ocampo-Friedmann, R. (2000) Ionizing-radiation resistance in the essication-tolerant cyanobacterium *Chroococcidiopsis*, *Applied and Environmental Microbiology*, **66**: 1489-1492

Bligh, E.G., Dyer, W.J. (1959) A rapid method for total lipid extraction and purification, *Canadian Journal of Biochemistry and Physiology*, **37**: 911-917

Bonente, G., Formighieri, C., Mantelli, M., Catalanotti, C., Giuliano, G., Morosinotto, T., Bassi, R. (2011) Mutagenesis and phenotypic selection as a strategy toward domestication of *Chlamydomonas reinhardtii* strains for improved performance in photobioreactors, *Photosynthetic Research*, **208**: 107-120

Bozarth, A., Maier, U-G., Zauner, S. (2009) Diatoms in biotechnology; modern tools and applications, *Applied Microbiology and Biotechnology*, **82**: 195-201

Brown, P. (2009) Algal biofuels research, development, and commercialization priorities: A commercial economics perspective, Gilbert: Diversified Energy (DEC000693).

Burczyk, J. (1987) Cell wall carotenoids in green algae which form sporopollenins, *Phytochemistry*, **26**: 121-128

Burczyk, J., Termińska-Pabis, K., Śmietana, B. (1995) Cell wall neutral sugar composition of Chlorococcalean algae forming and not forming acetolysis resistant biopolymer, *Phytochemistry*, **38**: 837-841

Burczyk, J., Smietana, B., Termińska-Pabis, K., Zych, M., Kowaloski, P. (1999) Comparison of nitrogen content amino acid composition and glucosamine content of cell walls of various Chlorococcalean algae, *Phytochemistry*, **51**: 491-497

Byrne, T. (2008) *pers.comm.*, 1st Nov 2008, The Roman Baths, Abbey Church Yard, Bath, BA1 1LZ, UK

Byrne, T. (2009) *pers.comm.*, 1st Mar 2009, The Roman Baths, Abbey Church Yard, Bath, BA1 1LZ, UK

Cadwell, G. (2012) *pers.comm.*, 13th Feb 2012, University College London, Darwin Building, Gower Street, London, WC1E 6BT, UK

Camacho Rubio, F., García Camacho, F., Fernández Sevilla, J.M., Chisti, Y., Molina Grima, E. (2002) A mechanistic model of photosynthesis in microalgae, *Biotechnology and Bioengineering*, **81**: 459-473

Campbell, N.A., Reece, J.B. (2002) *Biology.* 6th ed. Addison Wesley, San Francisco USA

Cao, J. (2009) A preliminary study of the effect of surface texture on algae cell attachment for a mechanical-biological energy manufacturing system, *Journal of Manufacturing Science and Engineering*, **131**: doi: 10.1115/bit.1.4000562

Carriquiry, M.A., Du, X., Timilsina, G.R. (2011) Second generation biofuels: economics and policies, *Energy Policy*, **39**: 4222-4234

Castenholz, R.W. (1969) Thermophillic blue-green algae and the thermal environment, *Bacteriological Reviews*, **33**:476-504

Castro-Ochoa, D., Peña-Montes, C., González-Canto, A., Alva-Gasca, A., Esquivel-Bautista, R., Navarro-Ocaña, A., Farrés, A. (2012) ANCUT2, an extracellular cutinase from *Aspergillus nidulans* induced by olive oil, *Applied Biochemistry and Biotechnology*, **166**: 1275-1290

Cha, T.S., Chen, J.W., Goh, E.G., Aziz, A., Loh, S.H. (2011) Differential regulation of fatty acid biosynthesis in two *Chlorella* species in response to nitrate treatments and the potential of binary blending microalgae oils for biodiesel application, *Bioresource Technology*, **102**: 10633-10640

Chaplin, M.F., Bucke, C. (1990) *Enzyme Technology*, Cambridge University Press: Cambridge, UK

Chen, F., Johns, M.R. (1994) Substrate inhibition of *Chlamydomonas reinhardtii* by acetate in heterotrophic culture, *Process Biochemistry*, **29**: 245-252

Chen, M., Tang, H., MA, H., Holand, T.C., NG, K.Y.S., Salley, S.O. (2011) Effect of nutrients of growth and lipid accumulation in the green algae *Dunaliella tertiolecta*, *Bioresource Technology*, **102**: 1649-1655

Chen, W., Sommerfeld, M., Hu, Q. (2011) Microwave-assisted nile red method for in vivo quantification of neutral lipids in microalgae, *Bioresource Technology*, **102**: 135-141

Cheng, Y-S., Zheng, Y., Labavitch, J.M., VanderGheynst, J.S., (2011) The impact of cell wall carbohydrate composition on the Chitosan flocculation of *Chlorella*, *Process Biochemistry*, **46**: 1927-1933

Chistenson, L., Sims, R. (2011) Review: production and harvesting of microalgae for wastewater treatment, biofuels and bioproducts, *Biotechnology Advances*, **29**: 686-702

Chisti, Y. (2008) Response to Reijnders: Do biofuels from microalgae beat biofuels from terrestrial plants?, *Trends in Biotechnology*, **26**: 351-352

Chisti, Y., Yan, J. (2011) Energy from algae: current status and future trends, *Applied Energy*, **88**: 3277-3279

Choi, G-G., Bae, M-S., Ahn, C-Y., Oh, H-M (2008) Induction of axenic culture of *Arthospira* (*Spirulina*) *platensis* based on antibiotic sensitivity of contaminating bacteria, *Biotechnology Letters*, **30**: 87-92

Chuck, C.J. (2007) *From cultivation to combustion – a scientific review of biodiesel*. Bath, UK.

Chuck, C.J. (2011) *pers.comm.*, May 2011, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Collet, P., Hélias, A., Lardon, L., Ras, M., Goy, R-A., Steyer, J-P. (2011) Life-cycle assessment of microalgae culture coupled to biogas production, *Bioresource Technology*, **102**:207-214

Comai, L., Henikoff, S. (2006) TILLING: practical single-nucleotide mutation discovery, *The Plant Journal*, **45**: 684-694

Cooney, M., Young, G., Nagle, N. (2009) Extraction of bio-oils from microalgae, *Separation and Purification Reviews*, **38**: 291-325

Cooper, M.S., Hardin, W.R., Peterse, T.W., Cattolico, R.A. (2010) Visualising 'green oil' in live algal cells, *Journal of Bioscience and Bioengineering*, **109**: 198-201

Cooper, R.M., (2012) *pers.comm.*, 4th May 2012, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Couderchet, M., Schmalfuβ, J., Böger, P. (1996) Incorporatio of oleic acid into sporopollenin and its inhibition by the chloroacetamide herbicide metazochlor, *Pesticide Biochemistry and Physiology*, **55**: 189-199

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G. (2005) Algae aquire vitamin B_{12} though a symbiotic relationship with bacteria, *Nature*, **438**: 90-93

Croft, M.T., Warren, M.J., Smith, A.G. (2006) Algae need their vitamins, *Eukaryotic Cell*, **5**: 1175-1183

Cuvelier, M.L., Oritz, A., Kim, E., Moehlig, H., Richardson, D.E., Heidelberg, J.F., Archibald, J.M., Worden, A.Z. (2008). Widespread distribution of a unique marine protistan lineage, *Environmental Microbiology*, **10**: 1621-1634

Danson, M.J. (2009) *pers.comm.*, 1st Jan 2008, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Day, J., Slocombe, S.P., Stanley, M.S. (2012) Overcoming biological constraints to enable the exploitation of microalgae for biofuels, *Bioresource Technology*, **109**: 245-251

de-Bashan, L.E., Bashan, Y. (2009) Immobilized microalgae for removing pollutants: review of practical aspects, *Bioresource Techology*, **101**: 1611-1627

Derenne, S., Largeau, C., Berkaloff, C., Rousseau, B., Wilhelm, C., Hatcher, P.G. (1992) Non-hydrolysable macromolecular constituents from outer walls of *Chlorella fusca* and *Nanochlorum eukaryotum*, *Phytochemistry*, **31**: 1923-1929

Demirbas, A. (2011) Biodiesel from oilgae, biofixation of carbon dioxide by microalgae: a solution to pollution problems, *Applied Energy*, **88**: 3541-3547

Demirbas, M.F. (2011a) Biofuels from algae for sustainable development, *Applied Energy*, **88**: 3473-3480

Demirbas, A., Demirbas, M.F. (2011) Importance of algae oil as a source of biodiesel, *Energy Conversion and Management*, **52**: 163-170

Devi, M.P., Mohan, S.V. (2012) CO_2 supplementation to domestic wastewater enhances microalgae lipid accumulation under mixotrophic environment: effect of sparging period interval, *Bioresource Technology*, **112**: 116-123

de Winder, B., Stal, L.J., Mur, L.R. (1990) *Crinalium epipsammum* sp. Nov.: a filamentous cyanobacterium with trichomes composed of elliptical cells and containing poly-β-(1,4) glucan (cellulose), *Journal for General Microbiology*, **136**: 1645-1653

Dinamarca, J., Shlyk-Kerner, O., Kaftan, D., Goldberg, E., Dulebo, A., Gidekel, M., Gutierrez, A., Scherz, A. (2011) Double mutation in photosystem II reaction centers and elevated CO_2 grant thermotolerance to mesophilic cyanobacterium, PLoS ONE 6(12): e28389. doi:10.1371/journal.pone.0028389

Dobritsa, A.A., Shestha, J., Morant, M., Pinot, F., Matsuno, M., Swanson, R., Lindberg Møller, B., Preuss, D. (2009) CYP704B1 is a long chain fatty acid ω-hydroxylase essential for sporopollenin synthesis in pollen of *Arabidopsis*, *Plant Physiology*, **151**: 574-589

Doughty, J. (2010) *pers.comm.*, 1st Mar 2010, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Dragone, G., Fernandes, B., Vicente, A.A., Teixeira, J.A. (2010) Third generation biofuels from microalgae, *Current research technology and education topics in applied microbiology and microbial biotechnology*, **2010**: 1355-1356.

Dunahay, T.G., Jarvis, E.E., Zeiler, K.G., Roessler, P.G., Brown, L.M. (1992) Genetic engineering of microalgae for fuel production, *Applied Biochemistry and Biotechnology*, **34/35**: 331-339

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K-H., Stackebrandt, E., (1992) The Prokaryotes (volume 4), 2nd ed, Springer, NY, USA

Eberley, J.O., Ely, R.L. (2012) Photosynthetic accumulation of carbon storage compounds under CO_2 enrichment by the thermophillic cyanobacterium *Thermosynechococcus elongatus*, *Journal of Industrial Microbiology and Biotechnology*, **39:** 843-850

Eberhard, S., Jain, M., Im, C.S., Pollock, S., Shager, J., Lin, Y., Peek, A.S., Grossman, A.R., (2006) Generation of an oligonucleotide array for analysis of gene expression in *Chlamydomonas reinhardtii*, *Current Genetics*, **49**: 106-124

Elsey, D., Jameson, D., Raleigh, B., Cooney, M.J. (2007) Fluorescent measurement of microalgal neutral lipids, *Journal of Microbiological Methods*, **68**: 639-642

El-Sheek, M.M., Hammouda, O.H., Kotkat, H. (1995) Photosynthesis, lipids and proteins in the cyanobacterium*Synechocystis* PCC 6803 as affected by temperature, *Biologia Plantarum*, **37**: 27-34

Estevez, M.S., Malanga, G., Puntarulo, S. (2001) Iron-dependant oxidative stress in *Chlorella vulgaris*, *Plant Science*, **161**: 9-17

Ferris, M.J., Hirsch, C.F. (1991) Method for isolation and purification of cyanobacteria, *Applied and Environmental Microbiology*, **57**: 1448-1452

Flynn, K.J., Greenwell, H.C., Lovitt, R.W., Shields, R.J. (2010) Selection for fitness at the individual or population levels: Modelling effects of genetic modifications in microalgae on productivity and environmental safety, *Journal of Theoretical Biology*, **263**: 269-280

Fuentes, C., Vanwinkle-Swift, K. (2003) Isolation and characterization of a cell wall-defective mutant of *Chlamydomonas monoica* (Chlorophyta), *Journal of Phycology*, **39**: 1261-1267

Galhano, V., Santos, H., Oliveira, M.M., Gomes-Laranio, J., Peixoto, F. (2011) Changes in fatty acid profile and antioxidant systems in a *Nostoc muscorum* strain exposed to the herbicide bentazon, *Process Biochemistry*, **46**: 2152-2162

Garcia-Pichel, F., Prufert-Bebout, L., Muyzer, G. (1996) Phenotypic and phylogenetic analyses show *Microcoleus chthonoplastes* to be a cosmopolitan cyanobacterium, *Applied and Environmental Microbiology*, **62**: 3284-3291

Gardner, R., Peters, P., Peyton, B., Cooksey, K.E. (2011) Medium pH and nitrate concentration effects on accumulation of triacylglycerol in two members of the Chlorophyta, *Journal of Applied Phycology*, **23**: 1005-1016

Gao, C., Xiong, W., Zhang, Y., Yuan, W., Wu, Q. (2008) Rapid quantitation of lipid in microalgae by time-domain nuclear magnetic resonance, *Journal of Microbiological Methods*, **75**: 437-440

Gaur, A.C., Pipes, W.O., Gotaas, H.B. (1960) Culturing *Oscillatoria* in organic wastes, *Water Pollution Control Federation*, **32**: 1060-1065

García Camacho, F., Molina Grima, E., Sánchez Mirón, A., González Pascual, V., Chisti, Y. (2001) Carboxymethyl cellulose protects algal cells against hydrodynamic stress, *Enzyme and Microbial Technology*, **29**: 602-610

García-Lepe, R., Nuero, O.M., Reyes, F., Santamaría, F. (1997) Lipases in autolysed cultures of filamentous fungi, *Letters in Applied Microbiology*, **25**: 127-130

Gibson D.G., Glass, J.I., Lartigue, C., Noskov, V.N., Chuang, R-Y., Algire, M.A., Benders, G.A., Montague, M.G., Ma, L., Moodie, M.M., Merryman, C., Vashee, S., Krishnakumar, R., Assad-Garcia, N., Andrews-Pfannkoch, C., Denisova, E.A., Young, L., Qi, Z-Q., Segall-Shapiro, T.H., Calvey, C.H., Parmar, P.P., Hutchinson III, C.A., Smith, H.O., Venter, J.C. (2010) Creation of a bacterial cell controlled by a chemically synthesized genome, *Science*, **329**: 52-56

González-López, C.V., Acién Fernández, F.G., Fernández-Sevilla, J.M., Sánchez Fernández, J.F., Molina Grima, E. (2012) Development of a process for efficient use of CO_2 from flue gases in the production of photosynthetic Microorganisms, *Biotechnology and Bioengineering*, **7:** 1637-1650

Gordon, J.M., Polle, J.E.W. (2007) Ultrahigh bioproductivity from microalgae, *Applied Microbiological Technology*, **76**: 969-975

Gow, N.A.R., Gadd, G.M. (1995) *The growing fungus*. Chapman and Hall, London UK

Gouviea, L., Marques, A.E., Lopes da Silva, T., Reis, A. (2009) *Neochloris oleabundans* UTEX #1185: a suitable renewable lipid source for biofuel production, *Journal of Industrial Microbiology and Biotechnology*, **36**: 821-826

Grant, I.F., Roger, P.A., Watanabe, I. (1985) Effect of grazer regulation and algal inoculation on photodependent nitrogen fixation in a wetland rice field, *Biology and Fertility of Soils*, **1**:61-72.

Greenwell, H.C., Laurens, L.M.L., Shields, R.J., Lovitt, R.W., Flynn, K.J. (2009) Placing microalghae on the biofuels priority list: a review of the technological challenges, *Journal of the Royal Society Interface*, **7**: 703-726

Gribovskaya, I.V., Kalacheva, G.S., Bayanova, Y.I., Kolmakova, A.A. (2007) Physiology-biochemical Properties of the cyanobacterium *Oscillatoria deflexa*, *Applied Biochemistry and Microbiology*, **45**: 285-290

Griffiths, M.J., Garcin, C., van Hille, R.P., Harrison, S.T.L. (2011) Interference by pigment in the estimation of microalgal biomass concentration by optical density, *Journal of Microbiological Methods*, **85**: 119-123.

Guarneiri, M.T., Nag, A., Smolinski, S.L., Darzins, A., Seibert, M., Pienkos, P.T. (2011) Examination of triacylglycerol biosynthetic pathways via de novo transcriptomic and proteomic analyses in an unsequenced microalga, *PLos One*, **6**: 1-13

Guzman, H.M., de la Jara, A.V., Duarte, L.C., Presmanes, K.F. (2010) Analysis of interspecific variation in relative fatty acid composition: use of flow cytometry to estimate unsaturation index and relative polyunsaturated fatty acid content in microalgae, *Journal of Applied Phycology*, **23**: 7-15

Haas, M.J., Wagner, K. (2011) Simplifying biodiesel production: The direct or in situ transesterification of algal biomass, *European Journal of Lipid Science and Technology*, **113**: 1219-1229

Hagen, C., Siegmund, S., Braune, W. (2002) Ultrastructural and chemical changes in the cell wall of *Haematococcus pluvialis* (Volvocales, Chlorophyta) during aplanospore formation, *European Journal of Phycology*, **37**: 217-226

Haik, Y., Selim, M.Y.E., Abdulrehman, T. (2011) Combustion of algae oil methyl ester in an indirect injection diesel engine, *Energy*, **36**: 1827-1835

Halim, R., Harun, R., Danquah, M.K., Webley, P.A. (2012) Microalgal cell disruption for biofuel development, *Applied Energy*, **91**: 116-121

Harith, Z.T., Yusoff, F.M., Mohammed, M.S., Shariff, M., Din, M., Ariff, A.B. (2009) Effect of different flocculants on the flocculation performance of microalgae *Chaetoceros calcitrans* cells, *African Journal of Biotechnology*, **8**: 5971-5978

Hatano, S., Joh, T., Miyamoto, T., Yoshimoto, M. (1992) Preparation of protoplasts from *Chlorella ellipsoidea* C-27, *Plant Cell Physiology*, **33**:651-655.

Hayat, M.A. (1986) *Basic techniques for transmission electron microscopy*. Academic Press: New York USA

He, L., Subramanian, V.R., Tang, Y.J. (2012) Experimental analysis and model-based optimization of microalgae growth in photobioreactors using flue gas, *Biomass and Bioenergy*, **41:** 131-138

Hejazi, M.A., Kleinegris, D., Wijffels, R.H. (2004) Mechanism of extraction of β-carotene from microalga *Dunaliellea salina* in two-phase bioreactors, *Biotechnology and Bioengineering*, **88**: 593-600

Hejazi, M.A., Wijffels, R.H. (2004) Milking of Microalgae, *Trends in Biotechnology*, **22**: 189-194

Henderson, R., Parsons, S., Jefferson, B. (2008) Review: The impact of algal properties and peroxidation on solid-liquid separation of microalgae, *Water Research*, **42**: 1827-1845

Hird, D.L., Worral, D., Hodge, R., Smartt, S., Paul, W., Scott, R. (1992). The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana* A6 gene displays similarity to β-1,3-glucanases, *The Plant Journal*, **4**:1023-1033

Honjoh, K., Suga, K., Shinohara, F., Maruyama, I., Miyamoto, T., Hatano, S., Iio, M. (2003) Preparation of protoplasts from Chlorella vulgaris K-73122 and cell wall generation of protoplasts from *C. vulgaris* K-73122 and C-27, *Journal of the Faculty of Agriculture, Kyushu University*, **47**: 257-266

Horn, S.J., Aasen, I.M., Østgaard, K.. (2000) Ethanol production from seaweed extract, *Journal of Industrial Microbiology and Biotechnology*, **25**: 249-254

Hulatt, C.J., Thomas, D.N. (2010) Dissolved organic matter (DOM) in microalgal photobioreactors: A potential loss in solar energy conversion?, *Bioresource Technology*, **101**: 8690-8697

Huang, C-C., Hung, J-J., Peng, S-H., Chen, C-N.N. (2012) Cultivation of a thermo-tolerant microalga in an outdoor photobioreactor: influences of CO_2 and nitrogen sources on accelerated growth, *Bioresource Technology*, **112**: 228-233

Illman, A.M., Scragg, A.H., Shales, S.W. (2000) Increase in *Chlorella* strains calorific values when grown in low nitrogen medium, *Enzyme and Microbial Technology*, **27**: 631-635.

Ishikawa, E., Sansawa, H., Abe, H. (2004) Isolation and characterization of a *Chlorella* mutant producing high amounts of chlorophyll and carotenoids, *Journal of Applied Phycology*, **16**: 385-393

Issa, A.A. (1999) Antibiotic production by the cyanobacteria *Oscillatoria angustissima* and *Calothix parietina*, *Environmental Toxicology and Pharmacology*, **8**: 33-37

Jaenicke, L., Kuhne, W., Spessert, R., Wahle, U., Waffenschmidt, S. (1987) Cell-wall lytic enzymes (autolysins) of *Chlamydomonas reinhardtii* are (hydroxy)proline-specific proteases, *European Journal of Biochemistry*, **170**: 485-491

Johnson, M.B., Wen, Z. (2010) Development of an attached microalgal growth system for biofuel production, *Applied Microbiology and Biotechnology*, **85**: 525-534

Johnson, M.B., Wen, Z. (2009) Production of biodiesel fuel from the microalga *Schizochytrium limacium* by direct transesterification of algal biomass, *Energy Fuels*, **23**: 5179-5183

Kaewpintong, K., Shotipruk, A., Powtongsook, S., Pavasant, P. (2007) Photoautotrophic high-density cultivation of vegetative cells of *Haematococcus pluvialis* in airlift bioreactor, *Bioresource Technology*, **98**: 288-295

Kaloudis, D. (2011) *pers.comm.*, 1st Dec 2011, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Kaloudis, D. (2012) *pers.comm.*, 15th Feb 2012, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Karatay, S.E., Dönmez, G. (2011) Microbial oil production from thermophile cyanobacteria for biodiesel production, *Applied Energy*, **88**: 3632-3635

Karseno, Harada, K., Bamba, T., Dwi, S., Mahakhant, A., Yoshikawa, T., Hirata, K. (2009) Extracellular phycoerythin-like protein released by freshwater cyanobacteria *Oscillatoria* and *Scytonema* sp., *Biotechnology Letters*, **31**: 999-1003

Kellaway, G.A. (1991) *Hot springs of Bath - Investigations of the thermal waters of the Avon valley*. Alden Press, Oxford UK

Kerney, R., Kim, E., Hangarter, R.P., Heiss, A.A., Bishop, C.D., Hall, B.K. (2011) Intracellular invasion of a green algae in a salamander host, *PNAS*, doi:10.1073/pnas.1018259108

Kerridge, E. (1967) *The agricultural revolution*. Routledge, Oxon UK.

Kobayashi, M., Hatta, K., Sugai, M. (1987) Endopolyploidy in *Chlorella*, *Archives of Microbiology*, **146**: 309-314

Kodner, R.B., Summons, R.E., Knoll, A.H. (2009) Phylogenetic investigation of the aliphatic, non-hydrolysable biopolymer algaenan, with a focus on green algae, *Organic Geochemistry*, **40**: 854-862

Kontkanen, H., Westerholm-Parvinen, A., Saloheimo, M., Bailey, M., Rättö, M., Mattila, I., Mohina, M., Kalkkinen, N., Nakari-Setälä, T., Buchert, J. (2009) Novel *Coprinopsis cinera* polyesterase that hydrolyses cutin and suberin, *Applied and Environmental Microbiology*, **75**: 2148-2157

Konur, O. (2011) The scientometric evaluation of the research on the algae and bioenergy, *Applied Energy*, **88**: 3532-3540

Kruskopf, M., du Plessis, S. (2006) Growth and filament length of the bloom forming *Oscillatoria simplicissima* (Oscillatoriales, Cyanophyta) in varying N and P concentrations, *Hydrobiologia*, **556**: 357-362

Kumar, A., Yuan, X., Sahu, A.K., Ergas, S.J., H., Van Langenhove, Dewulf, J. (2010) A hollow fibre membrane photobioreactor for CO_2 sequestration from combustion gas coupled with wastewater treatment: A process engineering approach, *Journal of Chemical Technology and Biotechnology*, **85**: 387-394

Kumar, S.S., Chounan, R.S., Thakur, M.S. (2010a) Trends in analysis of vitamin B_{12}, *Analytical Biochemistry*, **398**: 139-149

Kunjapur, A.M., Eldridge, R.B. (2010) Photobioreactor design for commercial biofuel production from microalgae, *Industrial and Engineering Chemistry Research*, **49**: 3516-3526

Lambert, J.A.M., Williams, E., O'Brien, P.A., Houghton, J.A. (1980) Mutation induction in the cyanobacterium *Gloeocapsa alpicola*, *Journal of General Microbiology*, **121**: 213-219

Lee, J-Y., Yoo, C., Jun, S-Y., Ahn, C-Y., Oh, H-M. (2010) Comparison of several methods for effective lipid extraction from microalgae, *Bioresource Technology*, **101**: S75-S77

Lee, A.K., Lewis, D.M., Ashman, P.J. (2009) Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel, *Journal of Applied Phycology*, **21**: 559-567

Leh, F., Posten, C. (2009) Closed photo-bioreactors as tools for biofuel production, *Current Opinion in Biotechnology*, **20**: 280-285

Leu, K-L., Hu, B-D. (2005) A programmed cell disintegration of *Chlorella* after heat stress, *Plant Science*, **168**: 145-152

Li, Y., Han, D., Hu, G., Dauville, D., Sommerfeld, M., Ball, S., Hu, Q. (2010) *Chlamydomonas* starchless mutant defective in ADP-glucose pyrophosphorylase hyper accumulates triacyleglycerol, *Metabolic Engineering*, **12**: 387-391

Li, Y., Han, D., Hu, G., Sommerfeld, M., Hu, Q. (2010) Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*, *Biotechnology and Bioengineering*, **107**: 258-268

Libertini, E., Li, Y., McQueen-Mason, S.J. (2003) Phylogenetic analysis of the plant endo-β-1,4-glucanase gene family, *Journal of Molecular Evolution*, **58**: 506-515

Liu, S., Liu, C., Huang, X., Chai, Y., Cong, B. (2006) Optimization of parameters for isolation of protoplasts from the Antartic sea ice alga *Chlamydomonas* sp. ICE-L, *Journal of Applied Phycology*, **18**: 783-786

Loppes, R. (1976) Release of enzymes by normal and wall-free cells of *Chlamydomonas*, *Journal of Bacteriology*, **128**: 114-116

Love, J. (2011) *pers.comm.*, 12th Jan 2011, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Luo, D., Hu, Z., Choi, D.G., Thomas, V.M., Realff, M.J., Chance, R.R. (2010) Life cycle energy and greenhouse gas emisions for an ethanol production process based on blue green algae, *Environmental Science and Technology*, **44**: 6870-8677

MacDonald, H. (2008) *pers.comm.*, 1st Oct 2008, University of the West of England, Coldharbour Lane, Bristol, BS16 1QY, UK

MacDonald, H. (2010) *pers.comm.*, 1st Jan 2010, University of the West of England, Coldharbour Lane, Bristol, BS16 1QY, UK

MacDougall, K.M., McNichol, J., McGinn, P.J., O'Leary, S.J.B., Melanson, J.E. (2011) Triacylglycerol profiling of microalgae strains for biofuel feedstock by liquid chomatography-high resolution mass spectrometry, *Analytical and Bioanalytical Chemistry*, **401**: 2609-2616

Malik, K.A. (1995) A convenient method to maintain unicellular green algae for long times as standing liquid cultures, *Journal of Microbiological Methods* **22**: 221-227

Malis-Arad, S., McGowan, R.E. (1982) A 'point of no return' in the cell cycle of *Chlorella*, *Plant and Cell Physiology*, **23**: 397-401

Mandalam, R.K., Palsson, B. ∅. (1997) Cell cycle of *Chlorella vulgaris* can deviate from the synchonous binary division model, *Biotechnology Letters*, **19**: 587-591

Markelova, A.G., Vladimirova, M.G., Kuptsova, E.S. (2000) A comparison of cytochemical methods for the rapid evaluation of microalgal viability, *Russian Journal of Plant Physiology*, **47**: 815-819

Markou, G., Georgakakis, D. (2011) Cultivation of filamentous cyanobacteria (blue-green algae) in agro-industrial wastes and wastewaters: a review, *Applied Energy*, **88**: 3389-3401

McCallum, C.M., Comai, L., Greene, E.A., Henikoff, S. (2000) Targeting induced local lesions in genomes (TILLING) for plant functional genomics, *Plant Physiology*, **123**: 439-442

McCann, C. (2009) *pers.comm.*, 1st Dec 2009, University College London, Darwin Building, Gower Street, London, WC1E 6BT, UK

McCarthy, S.S., Kobayashi, M.C., Niyogi, K.K. (2004) White mutants of *Chlamydomonas reinhardtii* are defective in phytoene synthase, *Genetics*, **168**: 1249-1257

McKendry, P. (2002) Energy production from biomass (part 1): overview of biomass, *Bioresource Technology*, **83**: 37-46

Melis, A. (2009) Solar energy conversion efficiencies in photosynthesis: Minimizing the chlorophyll antennae to maximize efficiency, *Plant Science*, **177**: 272-280

Mercer, P., Armenta, R.E. (2011) Developments on oil extraction from microalgae (review), *European Journal of Lipid Science Technology*, **113**: 539-547

Miller, S.R., Castenholz, R.W. (2000) Evolution of thermotolerance in hot spring cyanobacteria of the genus *Synechococcus, Applied and Environmental Microbiology*, **66**: 4222-4229

Miyake, M., Takase, K., Narato, M., Khatipov, E., Schnakenberg, J., Shirai, M, Kurane, R., Asada, Y. (2000) Polyhydroxybutyrate production from carbon dioxide by cyanobacteria, *Applied Biochemistry and Biotechnology*, **84-86**: 991-1002

Mohan, N., Hanumantha, R.P., Ranjinth, K.R., Sivasubramanian, V. (2010) Mass cultivation of *Choococcus turgidus* and *Oscillatoria* sp. and effective harvesting of biomass by low-cost methods, *Nature Precedings*, doi: 10.1038/bit.2010.4331.1

Moller, I., Sørensen, I., Bernal, A.J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J.D., Knox, J.P., Bacic, A., Willats, W.G.T. (2007) High-throughput mapping of cell-wall polymers within and between plants using novel microarrays, *The Plant Journal*, **50**: 1118-1128

Montero, M.F., Aristizábal, M., Reina, G.G. (2011) Isolation of high-lipid content strains of the marine microalga *Tetraselmis suecica* for biodiesel production by flow cytometry and single cell sorting, *Journal of Applied Phycology*, **23**: 1053-1057

Montsant, A., Zarka, A., Boussiba, S. (2001) Presence of a nonhydrolyzable biopolymer in the cell wall of vegetative cells and astaxanthin-rich cysts of *Haematococcus pluvialis* (Chlorophyceae), *Marine Biotechnology*, **3**: 515-521

Morweiser, M., Kruse, O., Hankamer, B., Posten, C. (2010) Developments and perspectives of photobioreactors for biofuel production, *Applied Microbiology and Biotechnology*, **87**: 1291-1301

Mutanda, T., Ramesh, D., Karthikeyan, S., Kumari, S., Anandraj, A., Bux, F. (2011) Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production, *Bioresource Technology*, **102**: 57-70

Nanjo, Y., Mizusawa, N., Wada, H., Slabas, A.R., Hayashi, H., Nishiyama, Y. (2010) Synthesis of fatty acids de novo is required for photosynthetic acclimation of *Synechocystis* sp. PCC 6803 to high temperature, *Biochimica et Biophysica Acta-Bioenergetics*, **1797:** 1483-1490

Němcová, Y., Kalina, T. (2000) Cell wall development, microfibril and pyrenoid structure in type strains of *Chlorella vulgaris*, *C.kessleri*, *C.sorokiniana* compared with *C.luteoviridis* (Trebouxiophyceae, Chlorophyta), *Algological Studies*, **100**: 95-105

Norsker, N-H., Barbosa, M.J., Vermuë, M.H., Wijffels, R.H.(2011) Microalgal production – a close look at the economics, *Biotechnology Advances*, **29**: 24-27

Northcote, D.H., Goulding, K.J., Horne, R.W. (1958) The chemical composition and structure of the cell wall of *Chlorella pyrenoidosa*, *Biochemistry Journal*, **70**: 391-402

Olivier, J.G.J., Janssens-Maenhout, G., Peters, J.A.H.W., Wilson, J.. (2011) *2011 Report: long-term trends in global CO_2 emissions*. PBL Netherlands Environmental Assessment Agency, The Hague, NL.

Ong, S-C., Kao, C-Y, Chiu, S-Y., Tsai, M-T., Lin, C-S. (2010) Characterisation of the thermal tolerant mutants of *Chlorella* sp. with high growth rate and application in outdoor photobioreactor cultivation, *Bioresource Technology*, **101**: 2880-2883

Ortiz-Marquez, J.C., Nascimento, M.D., de los Angeles Dublan, M., Curatti, L. (2012) Association with an ammonium-excreting bacterium allows diazotrophic culture of oil-rich eukaryotic microalgae, *Applied and Environmental Microbiology*, **78**: 2345-2352

Otero, A., Goto, K. (2005) Microalgae: the 'self-synchonized' eukaryotes, *Trends in Biotechnology*, **23**: 448-449

Packer, M. (2009) Algal capture of carbon dioxide; biomass generation as a tool for greenhouse gas mitigation with reference to New Zealand energy strategy and policy, *Energy Policy*, **37**: 3428-3437

Palmucci, M., Ratti, S., Giordano, M. (2011) Ecological and evolutionary implications of carbon allocation in marine phytoplankton as a function of nitrogen availability: a fourier transform infrared spectroscopy approach, *Journal of Phycology*, **47**: 313-323

Parmar, A., Singh, N.K., Pandey, A., Gnansounou, E., Madamwar, D. (2011) Cyanobacteria and microalgae: a positive prospect for biofuels, *Bioresource Technology*, **102**: 10163-10172

Park, J.B.K., Craggs, R.J., Shilton, A.N. (2011) Recycling algae to improve species control and harvest efficiency from a high rate algal pond, *Water Research*, **45**: 6637-6649

Park, M.O., Heguri, K., Hirata, K., Miyamoto, K. (2005) Production of alternatives to fuel oil from organic waste by the alkane producing bacterium, *Vibrio furnissi* M1, *Journal of Applied Microbiology*, **98**: 324-331

Parker, B. (2012) *pers.comm.*, 13th Feb 2012, University College London, Darwin Building, Gower Street, London, WC1E 6BT, UK

Pattanaik, B., Roleda, M.Y., Schumann, R., Karsten, U. (2008) Isolate-specific effects of ultraviolet radiation on photosynthesis, growth and mycosporine-like amino acids in the microbial mat-forming cyanobacterium *Microcoleus chthonoplastes*, *Planta*, **227**: 907-916

Patil, P.D., Gude, V.G., Manarswarmy, A., Cooke, P., Munson-McGee, S., Nirmalakhandan, N., Lammers, P., Deng, S. (2010) Optimisation of microwave assisted transesterification of dry algal biomass using response surface methodology, *Bioresource Technology*, **102**: 1399-1405

Patil, P.D., Gude, V.G., Manarswarmy, A., Cooke, P., Nirmalakhandan, N., Lammers, P., Deng, S. (2012) Comparison of direct transesterification of algal biomass under supercritical methanol and microwave irradiation conditions, *Fuel*, **97**: 822-831

Peng, C., Qi-Jie, C., Li-Ye, C., Hong-Bo, S. (2012) Some progress in sexual reproduction and sex determination of economic algae, *African Journal of Biotchnology*, **11:** 4706-4715

Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L.E., Bashan, Y. (2011) Review: Heterotrophic cultures of microalgae: metabolism and potential products, *Water Research*, **45**: 11-36

Pereira, H., Barriera, L., Mozes, A., Florindo, C., Polo, C., Duarte, C.V., Custódio, L., Varela, J. (2012) Microplate-based high throughput screening procedure for the isolation of lipid-rich-marine microalgae, *Biotechnology for Biofuels*, **4**: 61-72

Pienkos, P.T., Darzins, A. (2009)The promise and challenges of microalgal-derived biofuels, *Biofuels Bioproducts and Biorefining*, **3**: 431-440

Piganeau, G., Eyre-Walker, A., Grimsley, N., Moreu, H. (2011) How and why DNA barcodes underestimate the diversity of microbial eukaryotes, *PLoS ONE*, doi: 10.1371/bit.0016342

Pilon, L., Berberoğlu, H., Kandilian, R. (2011) Review: radiation transfer in photobiological carbon dioxide fixation and fuel production by microalgae, *Journal of Quantitative Spectroscopy and Radiative Transfer*, **112**: 2639-2660

Pio, T.F., Macedo, G.A. (2008) Cutinase production by *Fusarium oxysporum* in liquid medium using central composite design, *Journal of Industrial Microbiology and Biotechnology*, **35**: 59-67

Pio, T.F., Fraga, L.P., Macedo, G.A. (2008) Inoculum padronization for the production of cutinase by *Fusarium oxysporum*, *Journal of Industrial Microbiology and Biotechnology*, **39**: 74-77

Pistorius, A.M.A., Degrip, W.J., Egorova-Zachernyuk, T.A. (2009) Monitoring of Biomass composition from microbiological sources by means of FT-IR spectroscopy, *Biotechnology and Bioengineering*, **103**: 123-129

Pittman, J.K., Dean, A.P., Osundeko, O. (2011) The potential of sustainable algal biofuel production using wastewater sources, *Bioresource Technology*, **102**: 17-25

Polle, J.E.W., Kanakagiri, S., Jin, E.S., Masuda, T., Melis, A. (2002) Truncated chlorophyll antenna size of the photosystems – a practical method to improve microalgal productivity and hydrogen production in mass culture, *International Journal of Hydrogen Energy*, **27**: 1257-1264

Polle, J., Kanakagiri, S., Benemann, J.R., Melis, A. (1999) Maximizing photosynthetic efficiencies and hydrogen production by microalgal cultures, *Proceeding of the 1999 U.S. DOE Hydrogen Program Review*, NREL/CP-570-26938

Potter, U.J. (2009) *pers.comm.*, 1st Dec 2011, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Pouneva, I. (1997) Evaluation of algal culture viability and physiological state by fluorescent microscopic methods, *Bulgarian Journal of Plant Physiology*, **23**: 67-76

Prabakaran, P., Ravindran, A.D. (2011) A comparative study on effective cell disruption methods for lipid extraction from microalgae, *Letters in Applied Microbiology*, **53**: 150-154

Pulz, O. (2001) Photobioreactors: production systems for phototrophic microorganisms, *Applied Microbiology and Biotechnology*, **57**: 287-293

Pulz, O., Gross, W. (2004) Valuable products from biotechnology of microalgae, *Applied Microbiology and Biotechnology*, **65**: 635-648

Pulz, O., Trent, J., Shiraiwa, J. Weber, A., Schmid-Staiger, U., Appelbaum, S., Hezinger, S., Brandenburg, W.A., (2009) Performance and potential of phototrophic aquaculture: Microalgae, cyanobacteria and microcrops, *International algae congress*, 1-2 December 2009, Hotel Hafen, Hamburg, Germany

Purton, S., Stevens, D.R. (1997) Review: Genetic engineering of eukaryotic algae: progress and prospects, *Journal of Phycology*, **33**: 713-722

Purton, S. (2011) *pers.comm.*, 12th Jan 2011, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Purton, S. (2012) *pers.comm.*, 12th Sep 2012, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Qi, B. (2009) *pers.comm.*, 1st Mar 2009, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Qianqian, W., Shuang, M., Hong, X., Min, Z., Jingmin, C. (2011) Purification and the secondary structure of fucoidanase from *Fusarium* sp. LD8, *Evidence Based Complementary and Alternative Medicine*, **2011**: 8

Quinn, J., de Winter, L., Bradley, T. (2011) Microalgae bulk growth model with application to industrial scale systems, *Bioresource Technology*, **102**: 5083-5092

Radakovits, R., Jinkerson, R.E., Darzins, A., Posewitz, M.C. (2010) Genetic engineering of algae for enhanced biofuel production, *Eukaryotic Cell*, **9**: 486-501

Rahat, M., Reich, V. (1985) Correlations between characteristics of some free-living *Chlorella* sp. and their ability to form stable symbioses with *Hydra viridis*, *Journal of Cell Science*, **74**: 257-266

Raines, C.A. (2006) Transgenic approaches to manipulate the environmental responses of the C_3 carbon fixation cycle, *Plant Cell and Environment*, **29**: 331-339

Ratha, S.K., Prasanna, R. (2012) Bioprospecting microalgae as potential sources of 'green energy', challenges and perspectives, *Applied Biochemistry and Microbiology*, **48**: 109-125

Ray, S., Bagchi, S.N. (2002) Nutrients and pH regulate algicide accumulation in cutures of the cyanobacterium *Oscillatoria laetevirens*, *New Phytologist*, **149**: 455-460

Rayner, A.D.M., Griffith, G.S., Wildman, H.G. (1994) Differential insulation and the generation of mycelial patterns, *In*: D.S., Ingram, A.Hudson, ed. *Shape and form in plants and fungi*. London: The Linnean Society of London, pp.291-304

Razon, L.F., Tan, R.R. (2011) Net energy analysis of the production of biodiesel and biogas from the microalgae: *Haematococcus pluvialis* and *Nannochloropsis*, *Applied Energy*, **88**: 3507-3514

Reijnders, L. (2009) Transport Biofuels: Can they help limiting climate change without an upward impact on food prices?, *Journal of Consumer Protection and Food Safety*, **4**: 75-78

Rindi, F., Mikhailyuk, T.I., Sluiman, H.J., Friedl, T., López-Bautista, J.M. (2010) Phylogenetic relationships in *Interfilum* and *Klebsormidium* (Klebsormidiophyceae, Streptophyta), *Molecular Phylogenetics and Evolution*, **58**: 218-231

Rindi, F., Guiry, M.D., López-Bautista, J.M. (2008) Distribution, morphology and phylogeny of *Klebsormidium* (Klebsormidales, Charophyceae) in urban environments in Europe, *Journal of Phycology*, **44**: 1529-1540

Rodriguez, A., Perestelo, F., Carnicero, A., Regalado, V., Perez, R., de la Fuente, G., Falcon, M.A. (1996) Degradation of natural lignins and lignocellulosic substrates by soil-inhabiting fungi imperfecti, *FEMS Microbiology Ecology*, **21:**213-219

Rodríguez, M.C., Cerezo, A.S. (1996) The resistant 'biopolymer' in the cell walls of *Coelastrum sphaericum*, *Phytochemistry*, **43**: 731-734

Rodríguez, M.C., Noseda, M.D., Cerezo, A.S. (1999) The fibrillar polysaccharides and their linkage to algaenan in the trilaminar layer of the cell wall of *Coelastrum sphaericum* (Chlorophyceae), *Journal of Phycology*, **35**: 1025-1031

Rodríguez-García**, M.I.,** M'rani-Alaoui**, M.,** Fernández**, M.C.** (2003) Behaviour of storage lipids during development and germination of olive (*Olea europaea* L.) pollen, *Protoplasma*, **221**: 237-244

Rozema, J., Björn, L.O., Bornman, J.F., Gaberščik, A., Häder, D.P., Trošt, T., Germ, M., Klisch, M., Gröniger, A., Sinha, R.P., Lebert, M., He, Yniger, A., Sinha, R.P., Lebert, M., He, Y-Y., Buffoni-Hall, R., de Bakker, N.V.J., van de Staaij, J., Maijkamp, B.B. (2002) The role of UV-B radiation in aquatic and terrestrial ecosystems—an experimental and functional analysis of the evolution of UV-absorbing compounds, *Journal of Photochemisty and Photobiology B: Biology*, **66**: 2-12

Rühle, T., Hemschemeier, A., Melis, A., Happe, T. (2008) A novel screening protocol for the isolation of hydrogen producing *Chlamydomonas reinhardtii* strains, *BMC Plant Biology*, **8**: 107

Rusli, M.H. (2012) *pers.comm.*, 1st Jan 2012, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Salmon, E., van Duin, A.C.T., Lorant, F., Marquaire, P-M., Goddard III, W.A. (2009) Thermal decomposition in algaenan of *Botryococcus braunii* race L. Part 2: Molecular dynamics simulations using the ReaxFF reactive force field, *Organic Geochemistry*, **40**: 416-427

Sandefur, H.N., Matlock, M.D., Costello, T.A. (2011) Seasonal productivity of a periphytic algal community for biofuel feedstock generation and nutrient treatment, *Ecological engineering*, **37**: 1476-1480

Sanders, W.B., Moe, R.L., Ascaso, C. (2005) Ultrastructural study of the brown alga *Pteroderma maculiforme* (Phaeophyceae) in the free-living state and in lichen symbiosis with the intertidal marine fungus *Verrucaria tavaresiae* (Ascomycotine, European Journal of Phycology, **40**: 353-361

Sandesh Kamath, B., Vidhyavathi, R., Sarada, R., Ravishankar, G.A. (2008) Enhancement of carotenoids by mutation and stress induced carotenogenic genes in *Haematococcus pluvialis* mutants, *Bioresource Technology*, **99**: 8667-8673

Santos, H.M., Lodiero, C., Capelo-Martinezm J-L. (2009) *Ultrasound in chemistry: analytical applications*, Wiley: Weinheim, Germany

Saraf S, Thomas B. (2007) Influence of feedstock and process chemistry on biodiesel quality. *Process Safety and Environmental Protection*, **85**: 360-364

Sargeant, L. (2011) *pers.comm.*, 1st Aug 2011, The Roman Baths, Abbey Church Yard, Bath, BA1 1LZ, UK

Satoh, A., Kato, M., Yamato, K., Ishibashi, M., Sekiguchi, H., Kurano, N., Miyachi, S. (2011) Characterisation of the lipid accumulation in a new microalgal species, *Pseudochoricystis ellipsoidea* (Trebouxiophyceae), *Journal of the Japan Institute of Energy*, **89**: 909-913

Schlarb-Ridley, B. (2012) *pers.comm.*, 13th Feb 2012, University College London, Darwin Building, Gower Street, London, WC1E 6BT, UK

Scott, R. J., Stead, A.D. (1994) *Molecular and Cellular aspects of plant reproduction*. Cambridge University Press, Cambridge UK

Scott, R.J. (2008) *pers.comm.*, 1st Oct 2008, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J., Smith, A.G. (2010) Biodiesel from algae: challenges and prospects, *Current Opinion in Biotechnology*, **21**: 277-286

Scott, R.J. (2011) *pers.comm.*, 12thJan 2011, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Scheffer, M., Rinaldi, S., Gragini, A., Mur, L.R., Van Nes, E.H. (1997) On the dominance of filamentous cyanobacteria in shallow turbid lakes, *Ecological Society of America*, **78**: 272-282

Scragg, A.H., Illman, A.M., Carden, A., Shales, S.W. (2002) Growth of microalgae with increased calorific values in a tubular bioreactor, *Biomass and Bioenergy*, **23**: 67-73

Seckbach, J. (2007) *Algae and cyanobacteria in extreme environments.* Springer, Dordrecht NL

Shirvani, T., Yan, X., Inderwildi, O.R., Edwards, P.P., King, D.A. (2011) Life cycle energy and green house gas analysis for algae-derived biodiesel, *Energy and Environmental Science*, **4**: 3773-3778

Shivastav, A., Misha, S.K., Misha, S. (2010) Polyhydroxyalkanoate (PHA) synthesis by *Spirulina subsalsa* from Gujarat coast of India, *International Journal of Biological Macromolecules*, **46**: 255-260

Simpson, A.J., Zang, X., Kramer, R., Hatcher, P.G. (2003) New insights on the structure of algaenan from *Botryococcus braunii* race A and its hexane insoluble botryals based on multidimensional NMR spectroscopy and electrospray-mass spectrometry techniques, *Phytochemistry*, **62**: 783-796

Singh, J., Gu, S. (2010) Commercialisation potential of microalgae for biofuels production, *Renewable and Sustainable Energy Reviews*, **14**: 2596-2610

Singh, A., Nigham, P.S., Murphy, J.D. (2011) Mechanism and challenges in commercialization of algal biofuels, *Bioresource Technology*, **102**: 26-34

Singh, A., Nigam, P.S., Murphy, J.D. (2011a) Renewable fuels from algae: an answer to debatable land based fuels, *Bioresource Technology*, **102**: 10-16

Sivakumar, G., Xu, J., Thompson, R.W., Yang, Y., Randol-Smith, P., Weathers, P.J. (2012) Integrated green algal technology for bioremediation and biofuel, *Bioresource Technology*, **107**: 1-9

Smith, V.H., Sturm, B.S.M., Denoyelles, F.J., Billings, S.A. (2010) The ecology of algal biodiesel production, *Cell Press*, **25**: 301-309

Smith, A.G. (2011) *pers.comm.*, 12th Jan 2011, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Smith-Baedorf, H.D., Chuck, C.J., Mokebo, K.R., MacDonald, H., Davidson, M.G., Scott, R.J. (2012) Bioprospecting the thermal waters of the Roman Baths: Isolation of new species and analysis of the FAME profile for biodiesel production, *Applied Microbiology and Biotechnology*, (submitted for review)

Song, D., Fu, J., Shi, D. (2008) Exploitation of oil-bearing microalgae for biodiesel, *Chinese Journal of Biotechnology*, **24**: 341-348

Spanova, M., Daum, G. (2011) Squalene – biochemistry, molecular biology, process biotechnology and applications, *European Journal of Lipid Science and Technology*, **113**: 1299-1320

Spaulding, S.A. (2011) *pers.comm.*,[email], 1st Oct 2011, University of Colorado, Boulder, Colorado, USA

Spicer, A.P. (2012) *pers.comm.*, 13th Feb 2012, University College London, Darwin Building, Gower Street, London, WC1E 6BT, UK

Spiekermann, P., Rehm, B.H.A., Kalscheuer, R., Baumeister, D., Steinbüchel, A. (1999) A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds, *Archives of Microbiology*, **171**: 73-80

Stanghellini, C., Dai, J., Kempkes, F. (2011) Effect of near-infrared-radiation reflective screen materials on ventilation requirement, crop transpiration and water use efficiency of a greenhouse rose crop, *Biosystems Engineering*, **110**: 261-271

Stephens, E., Ross, I.L., King, Z., Mussgnug, J.H., Kruse, O., Posten, C., Borowitzka, M.A., Hankamer, B. (2010) An economic and technical evaluation of microalgal biofuels, *Nature Biotechnology*, **28**: 126-128

Subhadra, B.G. (2011) Water management policies for the algal biofuel sector in the southwestern United States, *Applied Energy*, **88**: 3492-3498

Sudesh, K., Taguchi, K., Doi, Y. (2001) Can cyanobacteria be a potential PHA producer?, *Focused on Ecomolecular Science Research*, **42**: 75-76

Sun, A., Davis, R., Starbuck, M., Amotz, A.B., Pate, R., Pienkos, P.T. (2011) Comparative cost analysis of algal oil production for biofuels, *Energy*, **36**: 5169-5179

Surek, B. (2008) Meeting report: algal culture collections 2008, *An International Meeting at the Culture Collection of Algae and Protozoa (CCAP)*, June 8-11, 2008, Dunstaffnage Marine Laboratory, Oban, United Kingdom. Protist, **159**: 509-517

Suriyamongkol, P., Weselake, R., Narine, S., Moloney, M., Shah, S. (2007) Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants – A review, *Biotechnology Advances*, **25**: 148-175

Suzuki, T., Yamaguchi, T., Ishida, M. (1997) Short Communication: Effects of some factors on protoplast formation of a microalga, *World Journal of Microbiology and Biotechnology*, **13**: 355-356

Svensen, Ø., Frette, Ø., Erga, S.R. (2007) Scattering properties of microalgae: the effect of cell size and cell wall, *Optical Society of America*, **46**: 5762-576

Takeda, H. (1988) Classification of *Chlorella* strains by cell wall sugar composition, *Phytochemistry*, **27**: 3823-3826

Takeda, H. (1993) Taxonomical assignment of chlorococcal algae from their cell wall composition, *Phytochemistry*, **34**: 1053-1055

Takeda, H., Hirokawa, T. (1978) Studies on the cell wall of *Chlorella* I. Quantitative changes in cell wall polysaccharides during the cell cycle of *Chlorella ellipsoidea*, *Plant and Cell Physiology*, **19**: 591-598

Tanaka, I. (1991) Micro-tubule-determined plastid distribution during microsporogenesis in *Lilium longiflorum*, *Journal of Cell Science*, **99**: 21-31

Tansey, M.R., Brock, T.D. (1972) The upper temperature limit for eukaryotic organisms, *The Proceedings of the National Acadamy of Sciences Online USA*, **69**: 2426-2428

Templier, J., Largeau, C., Casadevall, E. (1993) Variations in external and internal lipids associated with inhibition of the resistant biopolymer from the A race of *Botryococcus braunii*, *Phytochemistry*, **33**: 1079-1086

Timilsina, G.R., Csordás, S, Mevel, S. (2011) When does a carbon tax on fossil fuels stimulate biofuels?, *Ecological Economics*, **70**: 2400-2415

Tiwari, S. (2009) *pers.comm.*, 1st Mar 2009, The University of Bath, Claverton Down, Bath, BA2 7AY, UK

Thompson, G.A. (1996) Review: Lipids and membrane function in green algae, *Biochimica et Biophysica Acta*, **1302**: 17-45

Tripathi, U., Venkateshwaran, G., Sarada, R., Ravishankar, G.A. (2001) Studies on *Haematococcus pluvialis* for improved production of astaxanthin by mutagenesis, *World Journal of Microbiology and Biotechnology*, **17**: 143-148

Turner, P., Mamo, G., Karlsson, E.N. (2007) Potential and utilization of thermophiles and thermostable enzymes in biorefining, *Microbial Cell Factories*, doi: 10.1186/1475-2859-6-9

Uduman, N., Qi, Y., Danquah, M.K., Forde, G.M., Hoadley, A. (2010) Dewatering of microalgal cultures: a major bottleneck to algae-based biofuels, *Journal of Renewable and Sustainable Energy*, **2**: 1-15

Uduman, N., Bourniquel, V., Danquah, M.K., Hoadley, A.F.A. (2011) A parametric study of electrocoagulation as a recovery process of marine microalgae for biodiesel production, *Chemical Engineering Journal*, **174**: 249-257

Ueno, R. (2008) Visulaisation of sporopollenin-containing pathogenic green micro-algae *Prototheca wickerhamii* by fluorescent in situ hybridization (FISH), *Canadian Journal of Microbiology*, **55**: 465-472

Um, B-H., Kim, Y-S. (2009) Review: a chance for Korea top advance algal-biodiesel technology, *Journal of Industrial and Engineering Chemistry*, **15**: 1-7

Urano, N., Fuji, K. (2000) Effect of osmotic stabilizers on protoplast generation of *Chlorella ellipsoidea* yellow/white colour mutants, *Journal of Bioscience and Bioengineering*, **90**: 567-569

van Beilen, J.B. (2010) Why microalgal biofuels won't save the internal combustion machine, *Biofuels Bioproducts and Biorefining*, **4**: 41-52

Vandamme, D., Pontes, S.C.V., Goiris, K., Foubert, I., Pinoy, L.J.J., Muylaert, K. (2011) Evaluation of electrocoagulation-flocculation for harvesting marine and freshwater microalgae, *Biotechnology and Bioengineering*, **108**: 2320-2330

Vandamme, D., Foubert, I., Fraeye, I., Meeschaert, B., Muylaert, K. (2012) Flocculation of *Chlorella vulgaris* induced by high pH: role of magnesium and calcium and practical implications, *Bioresource Technology*, **105**: 114-119

Vandenbroucke, M., Largeau, C. (2007) Kerogen origin, evolution and structure, *Organic Geochemistry*, **38**: 719-833.

Vaara, T., Vaara, M., Niemlä, S. (1979) Two improved methods of obtaining axenic cultures of cyanobacteria, *Applied and Environmental Microbiology*, **38**: 1011-1014

Varfolomeev, S.D., Wasserman, L.A. (2011) Microalgae as source of biofuel, food, fodder and medicines, *Applied Biochemistry and Microbiology*, **47**: 789-807.

Vázquez-Martínez, G., Rodríguez, M.H., Hernández, F., Ibarra, J.E. (2004) Strategy to obtain axenic cultures from field collected samples of cyanobacterium *Phormidium animalis*, *Journal of Microbiological Methods*, **57**: 115-121

Wagner, L. (2007) Biodiesel from algae oil, *Mora Associates Research Report*, July 2007

Walker, T.L., Collet, C., Purton, S. (2005) Review: Algal transgenics in the genomic era, *Journal of Phycology*, **41**: 1077-1093

Walker, T.L., Purton, S., Becker, D.K., Collet, C. (2005a) Microalgae as bioreactors, *Plant Cell Reports*, **24**: 629-641

Walker, D.A. (2009) Biofuels, facts, fantasy and feasibility, *Journal of Applied Phycology*, **21**: 509-517

Wang, S-B., Chen, F., Sommerfeld, M., Hu, Q. (2005) Isolation and proteomic analysis of cell wall-deficient *Haematococcus pluvialis* mutants, *Proteomics*, **5**: 4839-4851

Wang, H., Laughinghouse, H.D., Anderson, M.A., Chen, F., Williams, E., Place, A.R., Zmora, O., Zohar, Y., Zheng, T., Hill, R. (2012) Novel bacterial isolate from Permian groundwater, capable of aggregating

potential biofuel-producing microalga *Nannochloropsis oceanica* IMET1, *Applied and Environmental Microbiology*, **78**: 1445-1453

Weyer, K.M., Bush, D.R., Darzins, A., Willson, B.D. (2010) Theoretical maximum algal oil production, *Bioenergy Research*, **3**: 204-213

Wijffels, R.H., Barbosa, M.J. (2011) An outlook on microalgal biofuels, *Science*, **329**: 796-802

Wilkie, A.C., Edmundson, S.J., Duncan, J.G. (2011) Indigenous algae for local bioresource production: Phycoprospecting, *Energy for Sustainable Development*, **15**: 365-371

Williams, P.J .le B., Laurens, L.M.L. (2010) Review: Microalgae as biodiesel and biomass feedstocks: review and analysis of the biochemistry, energetics an economics, *Energy and Environmental Science*, **3**: 554-590

Worrall, D., Hird, D., Hodge, R., Wyatt, P., Draper, J., Scott, R. (1992) Premature dissolution of the microsporocyte callose wall causes male sterility in transgenic tobacco, *The Plant Cell*, **4**: 759-771

Wyatt, N.B., Gloe, L.M., Brady, P.V., Hewson, J.C., Grillet, A.M., Hankins, M.G., Pohl P.I. (2012) Critical conditions for ferric chloride-induced flocculation of freshwater algae, *Biotechnology and Bioengineering*, **109**: 493-501

Xiong, F., Komenda, J., Kopecký. J., Nedbal, L. (1997) Strategies of ultraviolet-B protection in microscopic algae, *Physiologia Plantarum*, **100**: 378-388

Xu, H., Miao, X., Wu, Q. (2006) High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters, *Journal of Biotechnology*, **126**: 499-507

Yamada, K., Moriyama, R., Hatano, S. (1987) Production of osmotically labile cells in *Chlorella ellipsoidea* C-27 and C-102 by enzyme treatment, *Agricultural Biology and Chemistry*, **51**: 579-580

Yamada, T., Sakaguchi, K. (1981) Protoplast induction in *Chlorella* species, *Agricultural Biological Chemistry*, **45**: 1905-1909

Yamada, T., Sakaguchi, K. (1982) Electron microscopic studies of *Chlorella ellipsoidea* protoplast formation, *Journal of General Microbiology*, **128**: 1319-1327

Yamamoto, M., Fujishita, M., Hirata, A., Kawano, S. (2004) Regeneration and maturation of daughter cell walls in the autospore-forming green alga *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae), *Journal of Plant Research*, **117**: 257-264

Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y. (2011) Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance, *Bioresource Technology*, **102**: 159-165

Yang, X., Liu, P., Hao, Z., Shi, J., Zhang, S. (2012) Characterisation and identification of freshwater microalgal strains toward biofuel production, *Bioresources*, **7:** 686-695

Zhang, F., Cheng, L-H., Gao, W-L., Xu, X-H., Zhang, L., Chen, H.L. (2011) Mechanism of lipid extraction from *Botryococcus braunii* FACHB 357 in a biphasic bioreactor, *Journal of Biotechnology*, **154**: 281-284

Zhang, J., Hu, B. (2012) A novel method to harvest microalgae via co-culture of filamentous fungi to form cell pellets, *Bioresource Technology*, **114**: 529-535

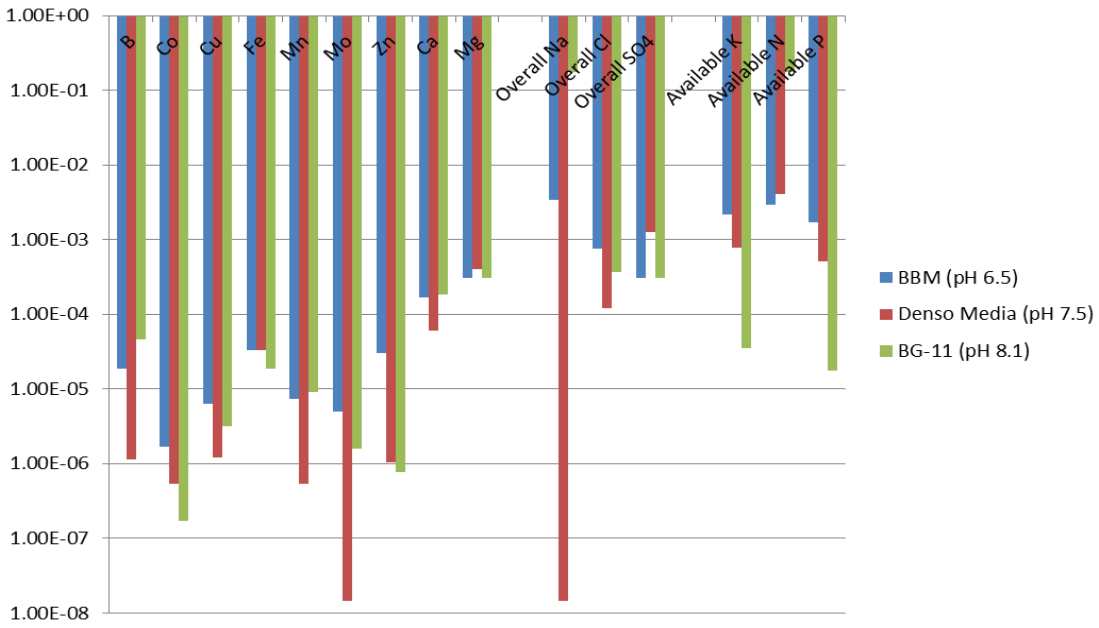
Zimmermann, W., Eeemüller, E., (1984) Degradation of Raspberry Suberin by *Fusarium solani* f. sp. *Pisi* and *Armillaria mellea*, *Journal of Phytopathology*, **110**: *192-199*

Zuppini, A., Andreoili, C., Baldan, B. (2007) Heat stress: as an inducer of programmed cell death in *Chlorella saccharophila*, *Plant Cell Physiology*, **48**: 1000-1009

Zych, M., Burczyk, J., Kotowska, M., Kapuścik, A., Banaś, A., Stolarczyk, A., Termińska-Pabis, K., Dudek, D., Klasik, S. (2009) Differences in staining of the unicellular algae Chlorococcales as a function of algaenan content, *Acta Agronomica Hungarica*, **57**: 377-381

10. APPENDICES

Appendix A: Nutrient concentrations for BBM and BG-11 culture media



Appendix B: FAME profiles (wt%) for WT and PM of *C.emersonii*

	BBM		BBM N-		BBM C+		BBM C+N-	
	WT	PM	WT	PM	WT	PM	WT	PM
C14:0	0.19	0	0.32	0.42	0.16	0.08	0.07	0.04
C15:0	0	0	0.09	0	0	0	0	0.03
C16:0	1.74	1.38	4.53	3.39	1.92	1.01	2.73	1.56
C16:1	0.17	0.29	0.25	0.25	0.10	0.08	0.26	0.25
C18:0	0.73	0.15	2.17	1.85	4.81	0.46	4.50	0.26
C18:1	2.39	0.84	8.18	7.38	1.67	0.76	4.56	2.87
C18:2	0.38	0.29	1.08	1.68	0.27	0.07	0.80	0.45
C18:3	2.75	3.01	3.92	2.39	1.20	0.43	4.32	1.96
C20:0	0	0	0.12	0.07	0.04	0	0.02	0
C20:1	0	0	0.18	0.17	0.05	0	0	0.08
C16:2	0	0.04	0.16	0.14	0	0	0.12	0.05
C16:3	0	0.09	0	0	0	0	0	0
C16:4	0.47	0.41	0	0	0	0.06	0	0
C18:4	0.21	0.27	0.33	0.31	0.12	0.09	0.39	0.33

Appendix C: Composition of the waters of the Roman Baths

Composition of the waters of the Great Bath (GB) and Kings Bath (KB) compared to historical measurements from the kings spring (Kellaway, 1991). Analysis performed by Severn Trent Services. Unless stated all data in mg l^{-1}.

Elements	**1874**	**1888**	**1936**	**1961**	**1979**	**1986**	**2011 GB**	**2011 KB**
B					0.59		0.47	0.51
Ca	377	402	392	392	382	390	394	421
Cu					0.002		0.002	0.003
Fe					0.88		0.25	0.39
Mg	47	52	51	54	53	58	51	53
Mn					0.068		0.135	0.140
Mo					<0.1		<0.002	<0.002
K	39	31	15.4	15.7	17.4	18.1	20.1	20.8
Na	129	135	177	174	183	228	196	203
Cl	280	277	274	276	287	335	332	330
NO_3^-N					<0.1			
HCO_3	86	88	193	216	192	187		
SiO_2					20.6 (Si)		42.99	42.27
SO_4	869	1061	1001	1021	1032	1030	978	977
BOD							<1	<1
pH					6.65			
Temp °C	46	47	49	48	45.3	43.5	39.0	45.0

Appendix D: 18S and 16S rDNA sequences of Roman Bath isolates

1 Coelastrella saipanensis full 18S rDNA sequence

TGCATGTCTAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGGTG
GTACCTTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCGTAAATCCCGACTTCTGGAAGGGACG
TATATATTAGATAAAAGGCCGACCGGACTTTGTCCGACCCGCGGTGAATCATGATATCTTCACGAAGCGCATG
GCCTTGTGCCGGCGCTGTTCCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGG
TGGTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGG
AAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACGGGGAGGTAGTGACAATAAATAACAATACCGGGCAT
TTCATGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCC
AGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTC
GGGTGGGTTCTAGCGGTCCGCCTATGGTGAGTACTGCTATGGCCTATCTTTCTGTCGGGGACGGGCTTCTGGGC
TTCACTGTCCGGGACTCGGAGTCGACGTGGTTACTTTGAGTAAATTAGAGTGTTCAAAGCAGGCTTACGCCCTG
AATACTTTAGCATGGAATAACACGATAGGACTCTGGCCTATCTTGTTTGGTCTGTAGGACTGGAGTAATGATTA
AGAGGGACAGTCGGGGGCATTCGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTACT
GCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTGGGGGCTCGAAGACGATTAGATACCG
TCGTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCGAATGTTTTTTCAATGACTTCGCCAGCACCTTAT
GAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGG
GCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAGGTCCAGACATAGTGA
GGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGGTTGCC
TTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTCCTAGTTGCTTTTTGCAGCTAGCTG
ACTTCTTAGAGGGACTATTGGCGTTTAGTCAATGGAAGTATGAGGCAATAACAGGTCTGTGATGCCCTTAGATG
TTCTGGGCCGCACGCGCGCTACACTGATGCATTCAACAAGCCTATCCTTGACCGAAAGGTCCGGGTAATCTTTG
AAACTGCATCGTGATGGGGATAGATTATTGCAATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAAGTC
ATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTGCTGGTGA
AGTGTTCGGATTGGCAGCTTAGGGTGGCAACACCTCAGGTCTGCCGAGAAGTTCATTAAACCCTCCCACCTAG
AGAAGAGA

2 Klebsormidium sp. full 18S rDNA sequence

NAGCCNTGCATGTCTAAGTATAAACTCTTTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTT
ATTTGATGGTACCTTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCACCAAATCCCGACTTCTG
GAAGGGACGTATTTATTAGATAAAAGGCCAATGCGGGCTTGCCCGGTATTGCGGTGAATCATGATAACTCGTC
GAATCGCACGGCCTTTGCGCTGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAG
GCCTACCATGGTGGTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC
CACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGACAATAAATAACA
ATGCTGGGCTTTTCAAAGTCTGGCAATTGGAATGAGTGCAATCTAAATCCCTCAACGAGGATCCATTGGAGGG
CAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTC
GTAGTTGGATTTTGGGTTGGGGCAGCCGGTCCGCCTCACGGTGTGCACCGGCTGACCCATCCTTCTTGCCGGGG
ACGCGCTCCTGGCCTTAACTGGTCGGGACGTGGAGTCGGCGATGTTACTTTGAAAAAATTAGAGTGTTCAAAG
CAGGCCTACGCTCTGAATACATTAGCATGGAATAACGTGATAGGACTCTGGTCCTATTGTGTTGGTCTTCGGGA
CCGGAGTAATGATTAATAGGGACAGTTGGGGATATTCGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTTAT
GAAAGACGAACCTTCTGCGAAAGCATTTATCAAGGATGTTTTCATTAATCAAGAACGAAAGTTGGGGGCTCGA
AGACGATCAGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCGGATGTTAATTTGTGAC
TCCGCCAGCACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAA
GGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCA
GGTCCAGACATAGTAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCT
TAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAACGAACGAGACCTCAGCCTGCTAACTAGTTACACGAAG
ATTCTTCTCCGTGGCCAACTTCTTAGAGGGACTATTTGGCGTCTACAGCCAATGGAAGTTTGAGGCAATAACAG
GTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGAATTCAACGAGTTTATAACCTGGGC
CGAAAGGTCTGGGTAATCTTGTGAAATTTCATGTGATGGGGATAGATTATTGCAATTATTAATCTTCAACGAGG
AATTCCTAGTAAGCGCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCC
TACCGATTGAATGATCCGGTGAAGTTTTCGGATTGCGGCTACTCCGGCGGTCCGCCGCCGAAGAAGCTGTGAG
AAGTTCATTAAACCTTATCANNAGAGAAGAGAAGTNGN

3 Hantzschia sp. full 18S rDNA sequence

AGCCATGCATGTCTAGTATAAATCTTTTACTTTGAAACTGCGAACGGCTCATTATATCAGTTATAGTTTATTTGA
TAGTCCCTTACTACTTGGATAACCGTAGTAATTCTAGAGCTAATACATGCGTCAATACCCTTCTGGGGTAGTAT
TTATTAGACTGAAACCAACCCCTTCGGGGTGATGTGGTGATTCATAATAAACGTGCGGATCGCATGCCTCTGGC
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4 Chroococcidiopsis thermalis full 16S rDNA sequence

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5 Mastigocladus laminosus full 16S rDNA sequence

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6 Microcoleus chthonoplastes full 16S rDNA sequence

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7 Oscillatoria sancta full 16S rDNA sequence

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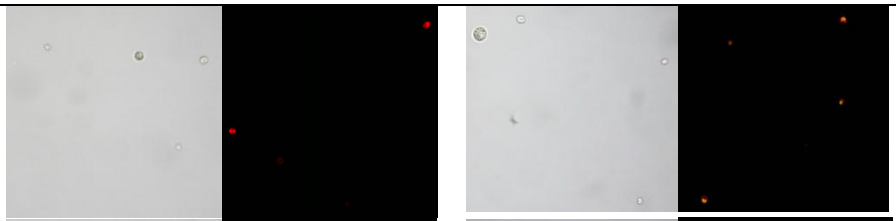
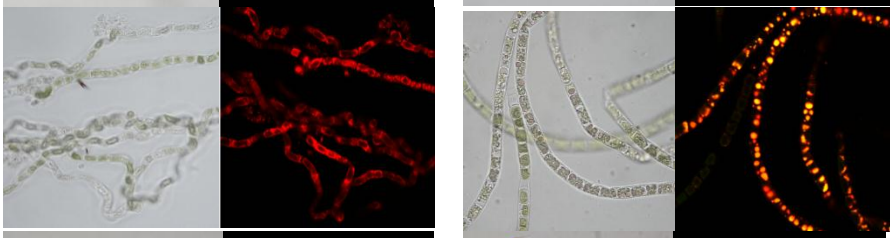
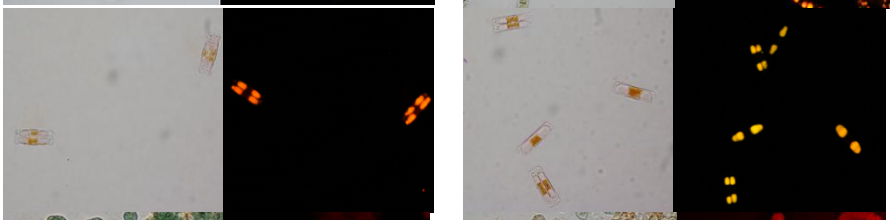
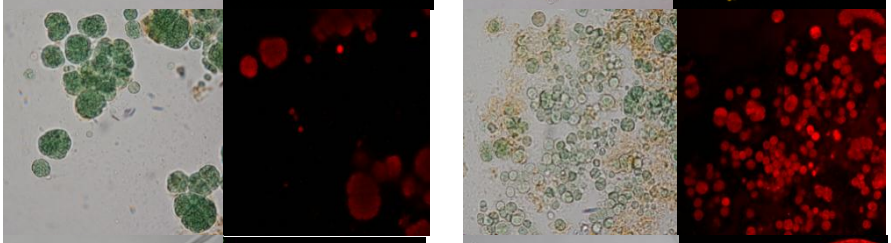
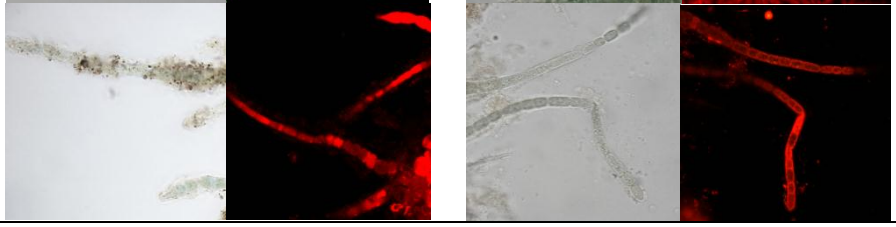
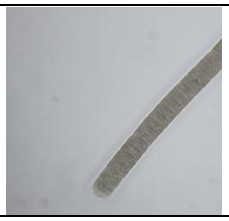
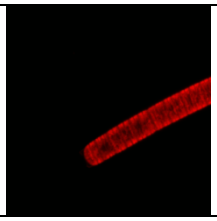
AAACN

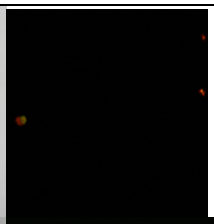
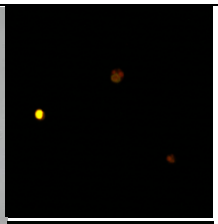
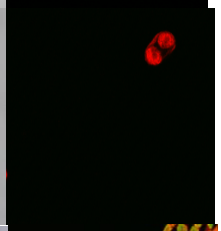
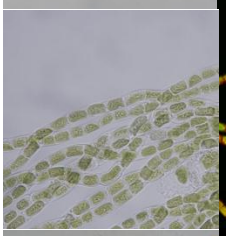
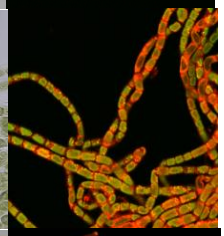
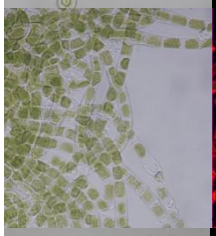
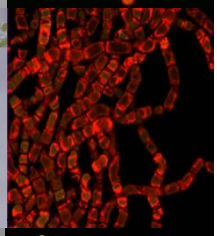
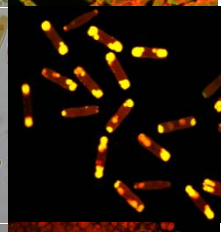
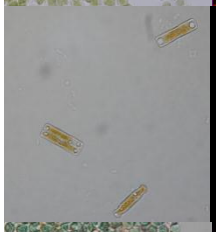
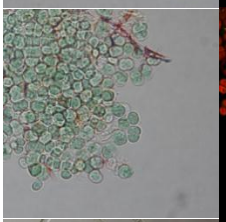
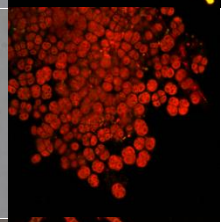
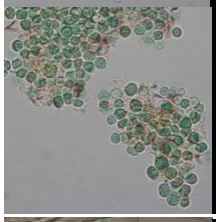
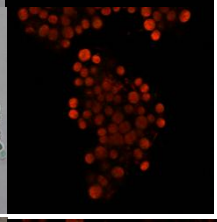
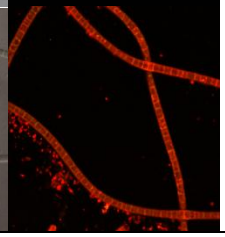
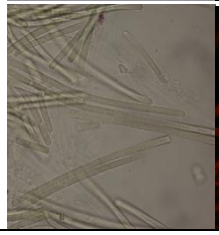
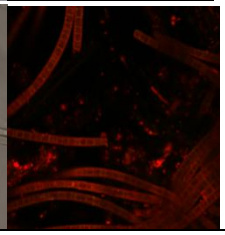
Appendix E: Light microscope and confocal images of Roman Bath isolates

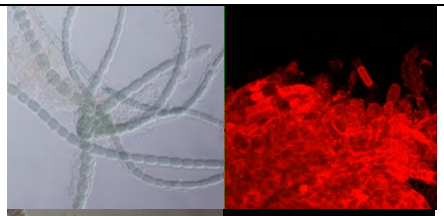
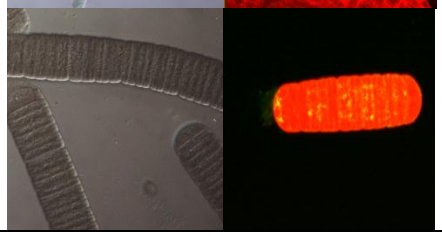
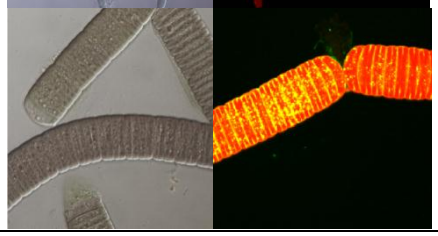
1 Images of C.emersonii and Roman Bath isolates stained with nile red after cultivation at 20°C with and without nitrogen starvation

Species	N sufficient	N starvation
Ce		
Cs		
K sp.		
H sp.		
Ct		
Mc		
Ml		

Os				

2 Images of C.emersonii and Roman Bath isolates stained with nile red after cultivation at 30°C with and without nitrogen starvation

Species	**N sufficient**		**N starvation**	
Ce				
Cs				
K sp.				
H sp.				
Ct				
Mc				

Ml		
Os		

3 Images of C.emersonii and Roman Bath isolates stained with nile red after cultivation at 40°C with and without nitrogen starvation

Species	**N sufficient**	**N starvation**
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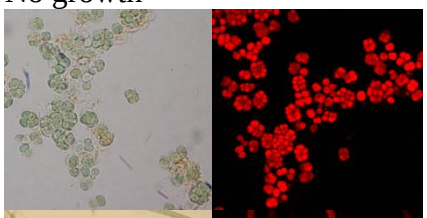
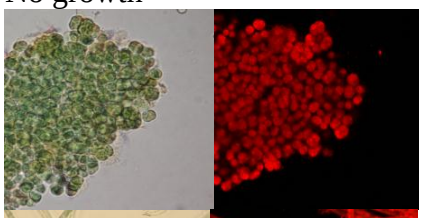
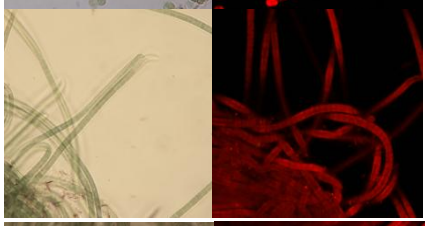
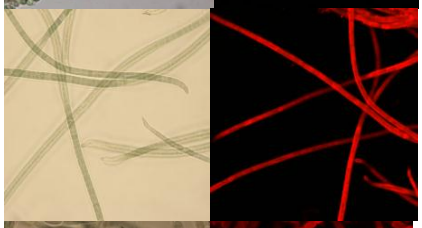
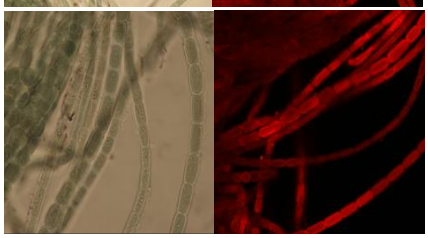
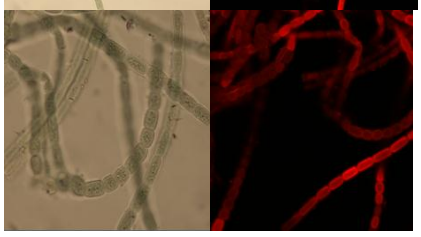
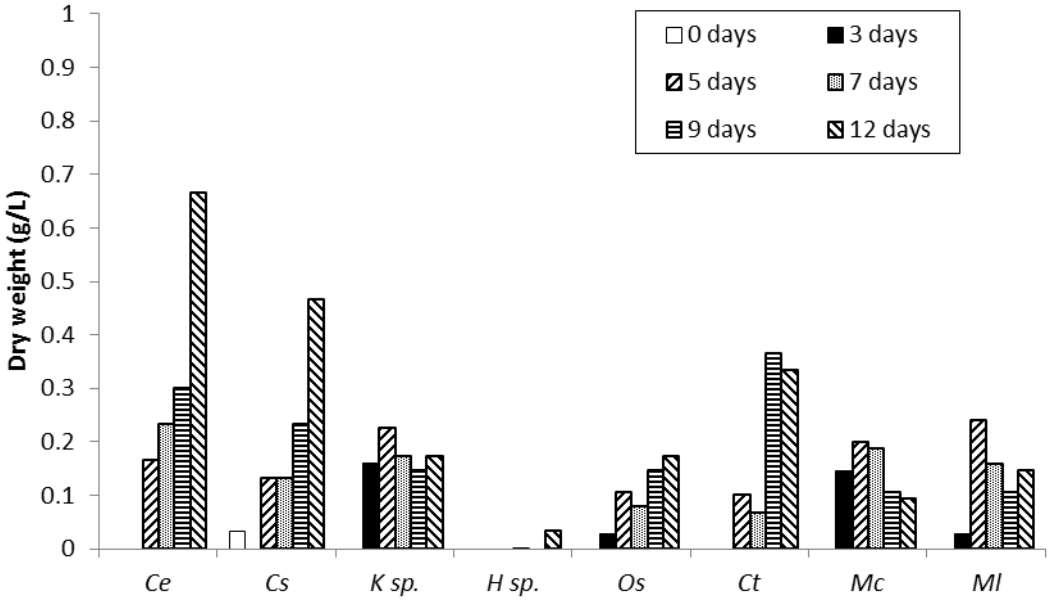
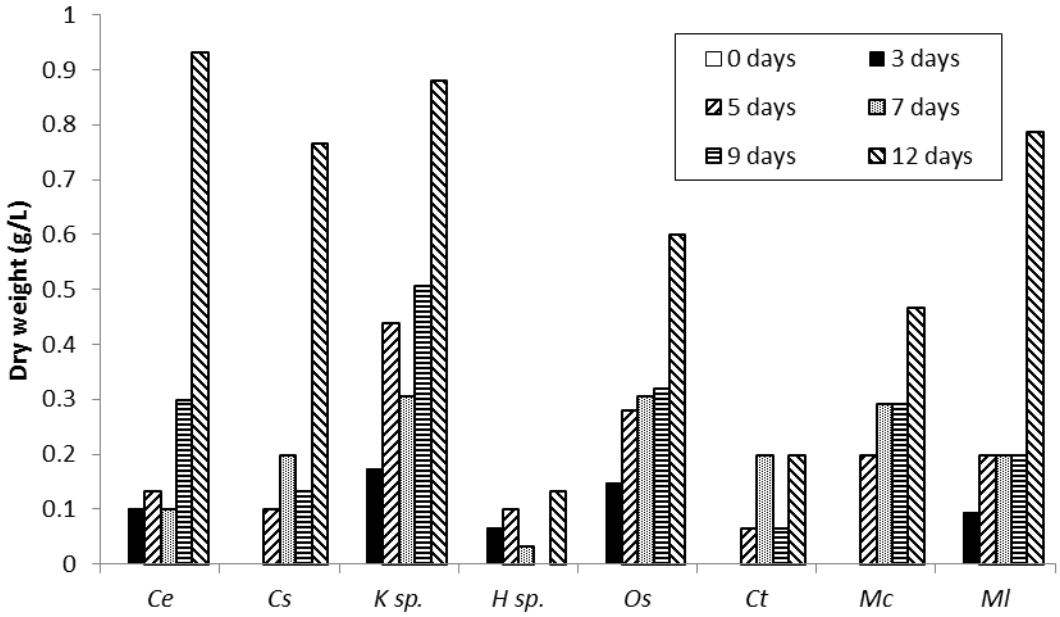
Cs	No growth	No growth
K sp.	No growth	No growth
H sp.	No growth	No growth
Ct		
Mc		
Ml		
Os	Poor growth	Poor growth

Appendix F: Growth and GCMS data for Roman Bath isolates

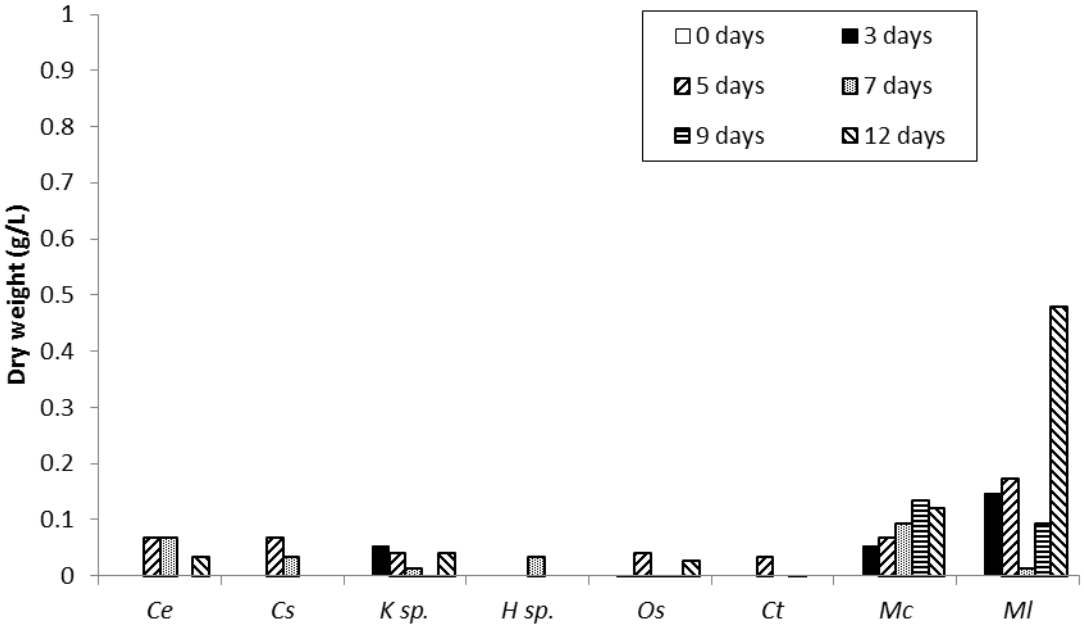
1 Dry weight of Bath isolates grown under nitrogen enriched conditions 20°C



2 Dry weight of Bath isolates grown under nitrogen enriched conditions at 30 °C



3 Dry weight of Bath isolates grown under nitrogen enriched conditions at 40 °C



4 FAME % profiles of the Roman Bath isolates cultured at 20 °C

	Ce		K sp.		Cs		H sp.		Os		Mc		Ml		Ct	
	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N
C14:0	0.2	0.6		1.1	0.3	0.8	5.1	5.6	5.2	4.1	2.8	2.7	4.0	3.4	1.6	5.2
C15:0	0.1	0.1				0.1		0.9								
C16:0	18.9	18.0	62.5	25.5	15.7	18.4	48.1	44.3	45.1	58.4	59.8	68.7	54.4	55.1	20.3	51.8
C16:1	1.4	1.4	7.7	0.7	5.7	4.4	35.2	32.4					11.1	8.8	15.1	21.1
C18:0	1.7	4.3	20.0	5.7	2.3	5.2	9.3	10.9	49.7	37.5	37.4	28.5	30.5	32.7		18.9
C18:1	28.5	38.5	3.8	2.7	28.1	30.0		4.7								
C18:2	10.2	6.9	5.9	46.2	8.9	6.2										
C18:3	34.7	23.0		15.4	32.8	24.8										
C20:1	0.0	0.0			0.4											
C20:4	0.0	0.0														
C16:2	3.0	2.0		0.6	3.1	2.3	2.3	1.26							62.9	3.0
C16:3	0.7	4.8		2.1	0.1	5.7										
C16:4	0.4	0.5			2.2	1.0										
C18:4	0.2	0.0			0.2	1.0										

5 FAME profiles (%) of the Roman Bath isolates cultured at 30 °C

	Ce		K sp.		Cs		H sp.		Os		Mc		Ml		Ct	
	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N	+N	-N
C14:0	0.3	0.5	2.4		0.4	0.5	5.0	4.3	30.2	20.5		0.8	1.8		5.0	4.7
C15:0	0.0	0.6			0.3	0.2									0.8	
C16:0	17.0	13.1	60.3	54.8	17.2	19.3	50.0	49.6	33.3	25.6	66.7	49.2	42.9	42.5	42.7	55.8
C16:1	2.1	3.8		3.7	4.7	3.3	20.3	18.5	12.6	2.3				23.3	24.3	26.6
C18:0	3.1	2.5	33.0	24.2	2.1	5.0	21.5	23.6	12.6	9.1	33.3	11.2	23.5	23.7	11.5	12.9
C18:1	8.7	21.1			19.4	16.5						8.7	31.7	10.5		
C18:2	9.1	12.8	4.3	13.6	11.8	10.8	3.2		10.9	5.6		5.0				
C18:3	46.6	29.2			28.7	32.6			0.4	34.6		16.0			14.4	
C20:1	0.0	1.0			0.5											
C20:4	1.4	0.0														
C16:2	1.7	3.1		3.8	4.6	3.6		4.0							1.3	
C16:3	0.9	3.5			7.8	8.3										
C16:4	6.7	5.9			0.9											
C18:4	2.4	2.8			1.6							9.0				

6 ***FAME profiles (%) of the microbes grown at 40 °C, rows given in italics are partial FAME profiles based on the limited amount of material***

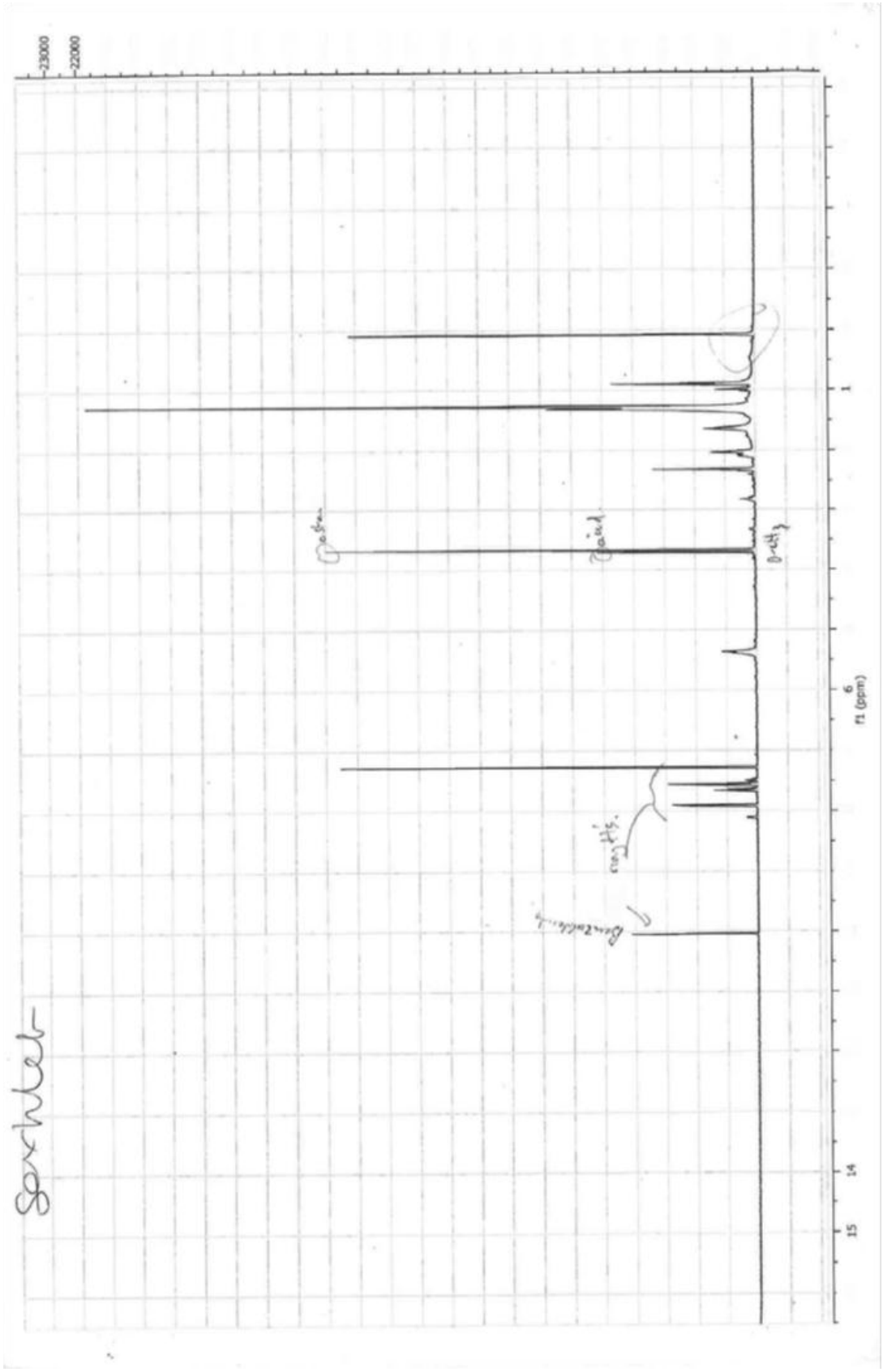
	Os		Mc		Ml		Ct	
	+N	-N	+N	-N	+N	-N	+N	-N
C14:0								
C15:0								
C16:0	54.4	56.1	49.0	49.1	38.5	42.2	56.7	54.8
C16:1					31.4	23.7		
C18:0	45.6	43.9	28.7	12.4	15.5	17.0	43.3	43.3
C18:1			6.2	9.6	14.5	16.1		
C18:2				8.9				
C18:3			16.0	20.0				

Appendix G: Extraction method GCMS data and NMR spectra

1 FAME profiles (wt%) for various extraction methods using C.emersonii

	AntonPaar 1	AntonPaar 2	Beadbeater	Microwave 1	Microwave 2	Sonication	Soxhlet
C13:0	0.02	0.01	0.03	0.03	0	0.04	0.03
C16:0	2.54	1.42	3.74	2.19	0.36	3.42	3.66
C16:1	0.27	0.17	0.42	0.21	0	0.34	0.42
C18:0	0.31	0.15	0.63	0.44	0.09	0.59	0.39
C18:1	4.15	2.77	6.78	3.39	0.42	5.73	6.97
C18:2	0.77	0.56	1.37	0.77	0.12	1.20	1.39
C18:3	1.55	2.28	4.86	2.73	0.45	3.67	5.35
C20:1	0.08	0.07	0.20	0.09	0	0.17	0.19
C16:2	0.10	0.09	0.22	0.14	0	0.20	0.23
C16:3	0.13	0.15	0.35	0.20	0	0.19	0.36
C16:4	0.27	0.39	0.80	0.44	0	0.65	0.91
C18:4	0.28	0.20	0.44	0.20	0.03	0.35	0.47

2 NMR spectra for C.emersonii extracted using the soxhlet method



3 NMR spectra for C.emersonii extracted using the Anton Paar 2 method